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*Scientific Edition*

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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## The Effect of Copper and Zinc Metals on Tincture of Digitalis and Tincture of Belladonna*,†

*By Anthony S. Ridolfo‡ and Earl P. Guth§*

Since the beginning of time man has made use of the medicinal properties of plants for the cure of his ailments. As the active agents in these plants became known, pharmacists endeavored to prepare concentrated or more usable forms of these active principles. During the course of the development of these preparations, a class of products known as tinctures was developed. Tinctures are generally described as hydroalcoholic solutions of the active constituents of crude drugs and are usually prepared by a process of percolation. In the preparation of tinctures, the pharmacist attempted to use a menstruum that would extract the active principles and as little of the medicinally inert material as possible. The elimination of the medicinally inert material is desired because it is of no value and it is known to undergo chemical and physical changes which make the finished preparation unsuitable for medicinal purposes. However, it is not possible under the conditions of percolation to obtain a solution of only the active principles. Inert material is always extracted. This material involves such compounds as carbohydrates, fats, proteins, saponins, chlorophyll, xanthophylls and carotenes.

That drug extractives are influenced by both physical and chemical factors has long been realized. Investigations are being conducted continually with the aim of increasing the activity of, and decreasing or preventing the deterioration of drug extractives. Some factors which have been studied embracing these are time, temperature, light, pressure and *p*H.

Much work has been done in recent years to perfect the process of extraction in order to eliminate inactive material from the finished product. Husa and Huyck (1) found that lengthening of a percolation tube would lead to a faster and more complete extraction of a drug with less menstruum. Also, preliminary maceration of drugs before percolation has been examined for efficiency in extracting drugs (2). The effect of variation in solvents on extraction of resins has been studied (3). That time of

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maceration and rate of flow are of importance in obtaining the active constituent of a drug has been brought forth by Husa and Huyck (4).

While encouraging results were obtained by much of this experimentation, it was apparent that there were other factors than those already studied which might influence the activity of drug extractives. Among these is included the effect of metals.

The possibility that metals might be useful in the preservation of drug extractives was suggested by their use in canned vegeable foods and in medicinal preparations. Leach and Winton (5) state that zinc salts have been used for greening peas. Zinc chloride is the salt used and a natural yellowish green tint is imparted when properly applied. This process has been kept secret. Jacobs (6) states that small traces of copper in canned peas will also improve their appearance.

The value of zinc in stabilizing insulin has led to the preparation of zinc protamine insulin. Zinc not only enhances the stability of the protamine insulin, but it also greatly prolongs the duration of the action of this insulin complex Copper is also used to preserve anesthetic ether. It acts here as an antioxidant and it prevents the formation of ethyl peroxides from ethyl oxide.

As far as we have been able to determine, metals have not been used to stabilize and improve the appearance of drug extractives, therefore a series of experiments was performed to determine the effect of metals in the stabilization of medicinals prepared by extraction of crude drugs. The study was made with two points in mind: 1. The preservation of the active principles of the preparation, and 2, the preservation of the color of the tincture.

## EXPERIMENTAL

**Experiment Number 1. Preparations of Tinctures.**—The investigation began with the preparation of several official tinctures. The tinctures were prepared according to the U. S. Pharmacopœia XI and National Formulary VI directions with the exception that the time of maceration was doubled.

Two hundred and fifty cubic centimeter portions of each tincture were placed in 8-fluid ounce screw-cap prescription bottles. To each portion of each tincture 5 Gm. of the following C. P. metals was added: copper, aluminum, zinc, nickel and lead. Two samples of each tincture were so prepared. One series was then stored in a dark cold room (4° C.) and the other series was stored in diffused light at room temperature (25° C.). Control samples (without metals) were stored under identical conditions.

The results of this experiment observed over a period of four months were striking, especially with Tincture of Belladonna and Tincture of Digitalis.

A striking effect noted at this time was the color change manifested in the Tincture of Belladonna and the Tincture of Digitalis with the copper and zinc metals. These tinctures gave a distinct green color, the density of which was measured with a Fisher Electrophotometer.

**Experiment Number 2. Determination of Concentration of Color.**—The tinctures to be examined photoelectrometrically were first clarified by centrifugalization at 1250 r. p. m. for fifteen minutes.

The procedure used to determine the concentration of color in the clarified tinctures was to take two and one-half cubic centimeters of tincture and dilute it to five cubic centimeters. The resulting solution was then viewed through a Fisher Electrophotometer using a 650 A. filter. The results of these readings are shown in Table II.

It may be seen from Table II that the intensity of the color of Tincture of Belladonna treated with zinc, which has a reading of 79.0 is much greater than the intensity of the color of the control Tincture of Belladonna with a reading of 47.0.

**Experiment Number 3. Passage of Tinctures through Metal to Produce a Color Change.**—It seemed desirable to ascertain whether the mere passage of a tincture through copper or zinc would also give rise to a color change. Therefore, a copper strainer was prepared from 10 Gm. of copper wire, and placed in the bottom of the percolator. The U. S. Pharmacopœia XI (7) methods were followed in preparing tinctures of Belladonna and Digitalis

TABLE I.—CHANGES IN TINCTURES STORED WITH 5 GM. OF METAL FOR FOUR MONTHS AT 4° C. AND 25° C.

| Tincture | Metal | Color | 4° C. Ppt. | Sp. Gr. | Color | 25° C. Ppt. | Sp. Gr. |
|---|---|---|---|---|---|---|---|
| Belladonna | Control | Dark green | + | 0.884 | Bright green | +++ | 0.880 |
| | Pb | Dark green | +++ | 0.884 | Bright green | +++ | 0.880 |
| | Al | Dark green | +++ | 0.884 | Bright green | +++ | 0.880 |
| | Zn | Dark green | +++ | 0.884 | Green | ++ | 0.880 |
| | Cu | Dark green | +++ | 0.884 | Green | ++ | 0.880 |
| | Ni | Dark green | +++ | 0.884 | Bright green | ++ | 0.880 |
| Digitalis | Control | Green[a] | + | 0.873 | Red brown | + | 0.873 |
| | Pb | Green | − | 0.873 | Red brown | − | 0.873 |
| | Al | Green | + | 0.873 | Red brown | + | 0.873 |
| | Zn | Green | + | 0.873 | Green | + | 0.873 |
| | Cu | Green | + | 0.873 | Green | + | 0.873 |
| | Ni | Green | + | 0.873 | Green | + | 0.873 |

[a] Developed a reddish fluorescence on standing.
− No ppt.; + slight; +++ heavy.

in this "coppered" percolator. The same procedure was followed substituting 10 Gm. of mossy zinc for the copper wire. Controls were prepared by manufacturing similar tinctures from the same sample of crude drugs omitting all metals. The color intensity of these tinctures was recorded by the electrophotometer and the results are shown in Table II. It was observed that the tinctures were affected by slight contact with metals. The time of contact in each case was forty-eight hours.

**Experiment Number 4. Introduction of Metallic Ions into the Tinctures.**—Since the presence of nonionized metal produced a color change, it was decided to investigate the effect of metallic ions on the tinctures. To 250-cc. portions of Tincture of Belladonna and Tincture of Digitalis, exact dilutions of copper ions in the form of copper sulfate and the zinc ions in the form of zinc sulfate were added.

A freshly prepared Tincture of Belladonna was then divided into nine portions of 250 cc. each.

TABLE II.—ELECTROPHOTOMETRIC EXAMINATIONS ON COLOR DENSITY OF TINCTURES

| Treatment | Readings: Tincture of Belladonna | Readings: Tincture of Digitalis |
|---|---|---|
| Stored 4 mo. with 5 Gm. metal: | | |
| Zn | 79.0 | 66.5 |
| Pb | 57.5 | 42.1 |
| Cu | 83.2 | 66.9 |
| Ni | 61.0 | 44.1 |
| Al | 63.0 | 57.5 |
| Control | 47.0 | 47.0 |
| Passed through 10 Gm. in the percolator: | | |
| Cu | 75.0 | 66.9 |
| Zn | 84.0 | 66.5 |
| Control | 65.0 | 52.9 |
| Introduction of metallic ions: | | |
| 8 p. p. m.[a] $(Cu)^{++}$ | 66.0 | 57.5 |
| 16 p. p. m. $(Cu)^{++}$ | 69.0 | 69.0 |
| 32 p. p. m. $(Cu)^{++}$ | 75.0 | 77.0 |
| 60 p. p. m. $(Cu)^{++}$ | 83.0 | 80.0 |
| 8 p. p. m. $(Zn)^{++}$ | 65.0 | 102.0 |
| 16 p. p. m. $(Zn)^{++}$ | 65.0 | 100.0 |
| 32 p. p. m. $(Zn)^{++}$ | 65.0 | 110.0 |
| 60 p. p. m. $(Zn)^{++}$ | 63.0 | 110.0 |
| Control | 63.0 | 52.0 |
| 5 Gm. added after 1 yr. storage: | | |
| Cu | 63 | 64.0 |
| Zn | [illegible] | 63.2 |
| Control | 44.0 | 43.8 |

[a] Parts per million.

These were placed in 8-ounce prescription bottles and to each of four samples 0.5 cc., 1 cc., 2 cc. and 5 cc. of the copper dilutions were added, respectively. To the other five, 0.5 cc., 1 cc., 2 cc. and 5 cc. of the zinc dilutions were added. This corresponded to a solution of the ions in a concentration of eight, sixteen, thirty-two and sixty parts per million.

A similar series was prepared with a freshly manufactured Tincture of Digitalis. These preparations were stored at room temperature in diffused light and daily observations for a change in color were made. The change in color intensity which occurred is shown in Table II.

TABLE III.—ASSAY OF DIGITALIS REFERENCE STANDARD POWDER BY OFFICIAL ONE HOUR FROG METHOD

(Dose = 0.005 cc./Gm. × dilution 1:3)

| Weight of Frog in Grams | Total Dose, Cc. | End Point |
|---|---|---|
| 24.42 | 0.3663 | S. S.[a] |
| 22.00 | 0.3300 | B.[b] |
| 28.52 | 0.4280 | S. S. |
| 18.85 | 0.2827 | S. S. |
| 21.40 | 0.3210 | S. S. |
| 20.85 | 0.3120 | B. |
| 23.77 | 0.3560 | B. |
| 24.77 | 0.370 | S. S. |
| 19.00 | 0.280 | S. S. |
| 19.20 | 0.290 | S. S. |

[a] S. S. = Systolic Standstill.
[b] B. = Beat.

TABLE IV.—FROG ASSAY OF TINCTURE OF DIGITALIS AFTER ONE YEAR OF STORAGE

(Ten frogs used for each assay dose)

| Sample | Dose, Cc./Gm. | % in Systolic Standstill | Relative Potency to Standard |
|---|---|---|---|
| Control | 0.005 | 90 | |
| | 0.006 | 60 | $\frac{0.005 \times 100}{0.0055} =$ |
| | 0.003 | 20 | |
| | 0.002 | 18 | 90.9% |
| | 0.0055 | 70 | |
| 5 Gm. Cu | 0.006 | 100 | |
| | 0.005 | 80 | $\frac{0.005 \times 100}{0.0048} =$ |
| | 0.003 | 30 | |
| | 0.002 | 30 | 104% |
| | 0.0048 | 70 | |
| 5 Gm. Zn | 0.005 | 60 | $\frac{0.005 \times 100}{0.006} =$ |
| | 0.007 | 90 | |
| | 0.006 | 70 | 83% |

**Experiment Number 5. Tests for the Presence of Metallic Ions in Treated Tinctures.**—Since there was a color change in the tinctures treated with copper zinc, copper sulfate and zinc sulfate, tests for the presence of the metallic ions were made.

**A. Test for Copper Ions—U. S. Pharmacopœia XI (8) Method.**—This test was performed with the Tincture of Digitalis treated with 5 Gm. of Copper metal, the Tincture of Digitalis containing 0.5 cc. of the dilution of copper sulfate. The same test was used on the corresponding Tincture of Belladonna. These tinctures with the added ions gave positive tests, while in all cases, those tinctures stored in contact with the copper metal gave negative results. This seemed to indicate that no metallic copper had entered the solution.

**B. Test (9) for Zinc.**—Ten cubic centimeters of each of the tinctures was evaporated to dryness over a water bath. The residue was then digested with 10 cc. of aqua regia until all the organic matter was decomposed. The aqua regia was then dissolved in distilled water and neutralized with ammonium hydroxide. Ten cubic centimeters of a 10% solution of potassium ferrocyanide was added. Zinc salts so treated yielded a white slimy precipitate of

zinc ferrocyanide. This precipitate was soluble in excess of the potassium ferrocyanide and insoluble in ammonium hydroxide. This test was used on the Tincture of Digitalis containing 5 Gm. of zinc metal, on the Tincture of Digitalis containing 0.5 cc. of the dilution of zinc sulfate, and on the corresponding Tincture of Belladonna. Only those tinctures treated with zinc sulfate gave positive tests for zinc.

**Experiment Number 6. Reproduction of Green Color in Faded Tinctures.**—The next experiment was to determine the power of the metals to reproduce a green color in tinctures which had become brown in appearance.

TABLE V.—ASSAY OF TINCTURE OF DIGITALIS BY THE U. S. P. XII CAT METHOD

| Sample | Kg. Weight of Cat | Dilution | Total Cc. Injected | Potency, Cc./Kg. |
|---|---|---|---|---|
| Control | 1.68 | 1–20 | 28.2 | 0.52 |
| | 1.30 | 1–15 | 18.0 | 1.2 |
| | 2.20 | 1–18 | 18.9 | 1.05 |
| | 1.90 | 1–20 | 15.0 | 0.79 |
| | 2.80 | 1–15 | 15.0 | 0.70 |
| Average | | | | 0.85 |
| 5 Gm. Cu | 1.4 | 1–20 | 24.0 | 0.85 |
| | 1.9 | 1–20 | 30.0 | 0.789 |
| | 2.2 | 1–20 | 41.0 | 0.905 |
| Average | | | | 0.84 |
| 5 Gm. Zn | 2.2 | 1–15 | 58.8 | 0.92 |
| | 2.2 | 1–15 | 34.0 | 0.60 |
| | 3.4 | 1–15 | 37.4 | 0.73 |
| Average | | | | 0.75 |

To 250 cc. each of Tincture of Belladonna and Tincture of Digitalis which had been stored at room temperature for a year and whose color had changed from a fluorescent reddish green to a brown color, 5 Gm. of zinc metal and 5 Gm. of copper metal, respectively, were added. These preparations were then placed at room temperature in diffused light and were observed for a period of four months. At the end of this time a definite green color reappeared in the tinctures. The intensity in color was recorded on an electrophotometer. From Table II, it may be seen that the green color of a faded tincture may be restored by storing the tincture with metal.

**Experiment Number 7. Effect of Metals in Stabilizing the Activity of Tinctures.**—Since the Tincture of Belladonna and the Tincture of Digitalis had undergone visible color changes, there was the possibility that the color changes might have been accompanied by a change in activity. This was investigated by assaying the tinctures by the official methods.

Both the U. S. Pharmacopœia XI (10) and the U. S. Pharmacopœia XII (11) assay methods for Tincture of Digitalis were performed on samples of the various tinctures. The potency of these tinctures was then compared with that of the U. S. Pharmacopœia Digitalis Reference Standard as was shown in Table III on the preceding page.

TABLE VI.—ASSAY FOR TOTAL ALKALOIDS OF TINCTURE OF BELLADONNA[a]

| Sample | Date of Manufacture | Assay Gm./100 Cc. | Assay after Two Years' Storage Gm./100 Cc. |
|---|---|---|---|
| Control | Jan. 24, 1941 | 0.058 | 0.057 |
| 5 Gm. Cu | Jan. 24, 1941 | 0.058 | 0.0537 |
| 5 Gm. Zn. | Jan. 24, 1941 | 0.058 | 0.0584 |
| Control | June 26, 1941 | 0.0415 | 0.0400 |
| 5 Gm. Cu | June 26, 1941 | 0.0415 | 0.0438 |
| 5 Gm. Zn | June 26, 1941 | 0.0415 | 0.0426 |

[a] Each assay shown is the average of five determinations.

A review of Table IV shows that no significant difference appeared in potency during this period of examination.

The U. S. Pharmacopœia XII method of assay on the various Tinctures of Digitalis were also undertaken at the expiration of another year's time. The results of these assays were recorded in Table V. They confirm the previous work on the assays by the frog method, for all samples of the tinctures maintained their potency. Neither the copper nor the zinc added to, or subtracted from, the potency of the various tinctures.

No physiological tests were carried out with the Belladonna Tinctures. However, the official quantitative alkaloidal assays were made. Table VI indicates that the alkaloidal content of Tincture of Belladonna is stable during two years' storage. Moreover, contact with copper or with zinc does not influence the alkaloidal content of Belladonna.

## SUMMARY AND CONCLUSIONS

1. Tincture of Belladonna and Tincture of Digitalis were treated under various conditions and the results tabulated. The most significant changes were produced by treating the tinctures with metallic zinc and metallic copper.

2. The investigation consisted of physical, chemical and biological assays to detect changes in appearance and activity of the drugs. It was shown that the metals, copper and zinc, did improve the stability of the color of the Tinctures of Digitalis and Belladonna without interfering with the potency of the active constituents of these preparations.

3. The Tinctures of Digitalis and Belladonna gave decided color changes when stored in contact with the zinc and copper metals. This is shown on Table I which points out the following:

(*a*) That the extent of color change could be measured with an electrophotometer.

(*b*) That the metal does not have to remain in contact with the tinctures for more than forty-eight hours before this color change is produced.

(*c*) That metals can reproduce green color in tinctures which have lost their color after being stored for one year. This means that the original color of the tincture is not stable and that introduction of copper or zinc metals to these tinctures help maintain the color.

(*d*) That a color change is brought about by minute traces of metallic ions.

4. Table V and Table VI clearly point out that the potency of the various tinctures of Belladonna and Digitalis were not impaired by the presence of copper and zinc metals.

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(8) *Ibid.*, 450.
(9) Bliss, A., "A Laboratory Manual of Qualitative Chemistry," ed. 3, George Banta Pub. Co., Menasha, Wis., 1924, p. 108.
(10) *Ibid.*, p. 7.
(11) The Pharmacopœia of the United States, Twelfth Revision, Mack Printing Company, Easton, Pa., 1942, p. 509.

# The Effect of Cetylpyridinium Chloride on the Bacterial Growth in the Oral Cavity*

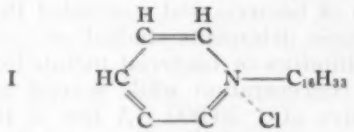
*By C. Lee Huyck*

Until recently it has not been possible to measure very accurately the efficiency of germicides in killing the bacteria in the oral cavity. Hanke (1) has developed a technique for measuring the bactericidal effectiveness of various germicides intended to be used in the oral cavity. This technique is based on the observation made by Miller (2) that numerous bacteria capable of producing acids from carbohydrates are always present in the oral cavity. The investigational work reported in this paper employs the technique of Hanke (1) in testing the effect of a solution of the germicide cetylpyridinium chloride on the bacterial growth in the oral cavity.

## PHYSICAL AND CHEMICAL PROPERTIES

Cetylpyridinium chloride[1] is a compound of high germicidal and bacteriostatic potency. Its chemical structure is shown in Formula I.

It is freely soluble in water, forming a clear, colorless solution. It is soluble in acetone and in alcohol but insoluble in ether and only slightly soluble in benzol. The aqueous solution is slightly acid to litmus and possesses a slight aromatic odor. The 1:1000 aqueous solution has an astringent taste which gradually disappears on dilution. When shaken, aqueous solutions foam like soap solutions because of their low surface tension.




The surface tension of aqueous isotonic solutions has been determined by the ring tensiometer method at 24.5° C. with the following results:

| | |
|---|---|
| 1:1000 cetylpyridinium chloride | 40.8 dynes/cm. |
| 1:5000 cetylpyridinium chloride | 45 dynes/cm. |
| 1:10,000 cetylpyridinium chloride | 51 dynes/cm. |
| Distilled water | 71.2 dynes/cm. |

Cetylpyridinium chloride may be considered as an invert soap differing from ordinary soap in containing no metallic base

* Received March 10, 1944, from The Wm. S. Merrell Company, Cincinnati, Ohio.

[1] Ceepryn brand of cetylpyridinium chloride manufactured by The Wm. S. Merrell Company, Cincinnati, Ohio.

and in being chemically reversed in respect to the electrical charges of the groups in the molecule. Its low surface tension gives it detergent and wetting properties which are desirable in wetting the skin and penetrating the tissues.

## MODE OF ACTION OF CETYLPYRIDINIUM CHLORIDE

According to the accepted theory of the detergency of wetting agents (3) cetylpyridinium chloride has both hydrophilic and lipophilic properties. The hydrophilic properties originate from the ionic properties of the salt while the lipophilic properties originate from long chain structure of the cetylpryidinium cation which is attracted to lipoids. Generally the higher the molecular weight of the hydrocarbon, the stronger is the lipophilic property. When cetylpyridinium chloride is placed in contact with water and lipoids, a definite orientation or arrangement establishes contact with both types of matter which is responsible for its high tissue penetrating action.

Frobisher (4) found that surface tension is an important factor in bacterial growth, morphology, cell division and to a lesser extent in the motility of bacteria as well as their ability to retain Gram's stain. The loss of virulence of pneumococci after treatment with surface tension reductants may be due to the fact that these bacteria disintegrate when they are subjected to low surface tension, and the dissolution of pneumococci by bile may be due to the low surface tension induced.

Baker, Harrison and Miller (5) studied the action of synthetic detergents on the metabolism of bacteria and concluded that all the cationic detergents studied are very effective inhibitors of bacterial metabolism at 1:3000 concentration while several are equally active at 1:30,000. A few of the anionic detergents inhibit the metabolism as effectively as the cationic compounds. Gram-positive and Gram-negative microorganisms are equally sensitive to the action of the cationic detergents, the inhibitory action of both types of detergents is influenced markedly by the hydrogen-ion concentration, and studies of homologous series of straight chain alkyl radicals show that maximum inhibition is exerted by lauryl, myristyl and cetyl radicals. Cationic detergents can exhibit an inhibitory activity comparable to quaternary ammonium compounds, and certain detergents especially anionic stimulate bacterial metabolism in low concentrations. In extending their work (6) the bactericidal action of a number of cationic and anionic germicidal detergents was studied. They concluded that the cationic detergents exhibit marked bactericidal effect on Gram-positive microorganisms and somewhat less pronounced action on Gram-negative organisms. The anionic detergents are germicidal only against the Gram-positive organisms and they are considerably less effective than the cationic compounds. The relationship between bactericidal action and chemical constitution is discussed.

Gershenfeld and Milanick (7) studied the bactericidal and bacteriostatic properties of surface-tension depressants and concluded that reduced surface tension alone does not explain the bactericidal action of the depressant but that the bactericidal action is due to a combination of three factors: (*a*) type of wetting agent, *i. e.*, anionic or cationic referring to the group which is responsible for the wetting property, (*b*) hydrogen-ion concentration of the environment, and (*c*) specific organism or groups of organisms of both Gram-positive and Gram-negative groups against which the wetting agent is tested.

Albert (8) has reviewed the methods by which various types of antiseptics kill bacteria. Cationic germicidal wetting agents, of which cetylpyridinium chloride is an example, contain basic ions or cations which interact with the acid groups of the bacteria to form feebly ionized compounds.

## QUATERNARY AMMONIUM COMPOUNDS AS GERMICIDES

As early as 1916 Jacobs and his co-workers (9, 10, 11) at the Rockefeller Institute reported the results of a systematic study of synthetic germicides. They pointed out that the quaternary ammonium salts have definite germicidal action, but until recently this type of compound has been very little investigated.

In a brief review of quaternary compounds used as disinfectants, Wakelin (12) reported that the cetyl radical is instrumental in rendering these compounds surface active making them good wetting agents. Quaternary compounds containing sulfur are well known in Great Britain as disinfecting and preserving agents for preparations containing sugar, starch and protein. A nutrient gelatin mixture containing dextrose preserved with 0.05 per cent of a quaternary compound containing sulfur did not decompose after ninety days.

Shelton, Van Campen and Nisonger (13) correlated the structure and germicidal activity of certain acyclic quaternary am-

monium salts. In testing the effect of the chain length of the alkyl group on the germicidal activity of alkyl quaternary ammonium chlorides, they found that the germicidal activity increased rapidly from an alkyl group containing eight carbon atoms to an alkyl group containing sixteen carbon atoms. With alkyl radicals containing more than sixteen carbon atoms the germicidal activity gradually decreased. In tests on the effect of various halogen anions of cetylpyridinium halides for germicidal activity, the chloride was slightly more active than the bromide and the bromide was more active than the iodide. Also, the sulfate anion was not quite as germicidal as the chloride anion. The organic anions such as the acetate, benzoate and salicylate were active germicides but they were less active than the inorganic anions.

In searching for a compound which can effectively inhibit the metabolism of microorganisms found in the lesions of dental caries or in plaques associated with such lesions, Miller, Baker and Harrison (14) studied the action of the quaternary ammonium type wetting agent described by Domagk (15) on the metabolism of bacteria associated with dental caries. The inhibition of metabolism of the so-called dental bacteria was greater than the inhibition of metabolism obtained by Miller (16) for fluoride or iodoacetate. Because of the unusual inhibitory action of the quaternary ammonium compound and its excellent power of penetration and cleansing, as well as its low toxicity for the mucous membranes, the authors concluded that it deserves further study.

Miller and Baker (17) reported on the comparative effects of a number of anionic and cationic detergents on the metabolism of Gram-positive and Gram-negative bacteria. They found the anionic detergents inhibited the metabolism of Gram-positive bacteria without having any significant effect on the Gram-negative bacteria. On the contrary, the cationic detergents inhibited the metabolism of both the Gram-positive and Gram-negative bacteria to the same degree.

In looking for a nontoxic compound possessing the property of rapid penetration of the dense mass surrounding the teeth, Miller, Muntz and Bradel (18) tested the inhibitory effect of the quaternary ammonium synthetic detergent described by Domagk (15). This compound reduced the surface tension of water in very low concentrations and it acted as a wetting and cleansing agent. Besides suppressing very effectively aerobic glycolysis of plaque material, it prevented any significant decrease in *p*H of this material. *In vivo*, the results demonstrated that the lactic acid bacteria were completely suppressed. In view of the present state of knowledge of dental caries, however, no definite conclusions concerning the prevention or treatment of dental caries can be drawn from this work. The efficacy of any agent or procedure proposed for the cure, prevention or mitigation of dental caries must be determined only on the basis of results obtained from clinical studies.

Shelton, Van Campen, Tilford and Nisonger (19) extended their work on the correlation of structure and germicidal activity of some quaternary ammonium salts by reporting on compounds derived from cyclic amines. Pyridine, picoline, lutidine, quinoline, piperidine, pipecoline and methyl morpholine were the cyclic amines tested. The maximum bactericidal activity was produced by the pyridine derivative, cetylpyridinium chloride. The cetyl amines derived from morpholine and piperidine had little germicidal action, but the quaternary salts obtained from the *N*-methyl amines were strongly germicidal. *N*-Cetyl-*N*-methyl piperidinium bromide was equal to cetylpyridinium chloride in bactericidal activity, followed in decreasing order by the quinolinium salt and the *N*-methyl morpholinium salt. It was concluded that the compounds having the simplest structure about the nitrogen atom had the highest germicidal activity. Generally, C-alkylation of the cyclic amine nucleus reduced the germicidal activity, and in the same way an increase in the molecular weight of the cyclic amine had a negative effect. The presence of oxygen in the molecule decreased the activity as suggested by the inactivity of the morpholinium salt as compared to the piperidinium salt.

Blubaugh, Botts and Gerwe (20) found that both the tincture and the aqueous solution of cetylpyridinium chloride were highly germicidal for virulent organisms in the presence and absence of organic matter when tested by the F. D. A. technique. Germicidal activity was determined for several species of pathogenic and nonpathogenic organisms. The bactericidal properties of this compound compared favorably with those of other well-known germicides of the mercurial, phenolic and halogenous types.

Green and Birkeland (21) tested the germicidal activity of cetylpyridinium chloride on the more common spores by the F. D. A. technique in liquid media and on contaminated instruments. The results indicated that cetylpyridinium chloride was

an effective and practical germicide for the more common bacterial spores.

Blubaugh, Gerwe, Botts and Helwig (22) continued the study of the germicidal activity of cetylpyridinium chloride on various organisms by the F. D. A. method and compared the results with a series of well-known germicides. Test for bacteriostatic activity demonstrated no inhibition in the case of vegetable cells but, on the contrary, the results indicated that for periods in excess of twenty-four hours, the action was bactericidal rather than bacteriostatic. Tests for killing time showed that a 1:4000 dilution of the compound was bactericidal for vegetative cells in less than fifteen seconds.

In testing cetylpyridinium chloride pharmacologically, Warren, Becker, Marsh and Shelton (23) found a 1:3000 solution slightly irritating to the conjunctival mucous membrane but not to the skin. When injected intravenously in rabbits the M. L. D. was 20 mg. per Kg. It was more toxic when given intraperitoneally. Given by mouth 400 mg. per Kg. killed one rabbit in six. Daily oral administration of smaller doses to rabbits for five weeks caused no significant damage. The toxic manifestations were curare-like action with central stimulation and peripheral paralysis.

Sarber (24) compared the effectiveness of cetylpyridinium chloride with sodium ethylmercuri-thiosalicylate and hexylresorcinol. He showed that cetylpyridinium chloride had the highest relative efficiency of the three germicides tested. Sodium ethylmercuri-thiosalicylate and hexylresorcinol followed in effectiveness in the order given. The author advanced evidence to support the theory that the skin has a definite bactericidal power which it may exert if it is not injured by chemicals. With germicides of low toxicity like cetylpyridinium chloride the combined action of the two agents overcome bacterial invasion which neither the skin alone nor the germicide was able to overcome by itself.

Clarke (25) made a bacteriological comparison of cetylpyridinium chloride with other sterilizing agents. No skin reaction followed its use. Scratch and patch tests of the 1:200 tincture and an aqueous solution were negative at the end of twenty-four and forty-eight hours as well as seven days.

Green and Birkeland (26) determined the effect of phenol, iodine and cetylpyridinium chloride solutions on experimental *Staphylococcus aureus* infections of the choric-allantoic membrane of chick embryos. Cetylpyridinium chloride definitely reduced the degree of infection. Solutions of phenol and iodine had no appreciable effect.

Ordal and Borg (27) found that the lactic dehydrogenase of *S. aureus* was inhibited by $1.7 \times 10^{-4}$ *M.* cetylpyridinium chloride. A concentration of $1.1 \times 10^{-3}$ *M.* cetylpyridinium chloride was required to inhibit the lactic dehydrogenase of *Escherichia coli*. Both cationic and anionic wetting agents inhibited the oxidation of lactate solutions by *S. aureus* but only the cationic agent inhibited the oxidation of lactate solution by *E. coli*.

Kolloff, Wyss, Himelick and Mantele (28) tested a group of 27 alkylpyridinium and alkyl-picolinium halides. Preliminary germicidal tests were made to discover any increase in activity due to the introduction of a methyl radical into the aromatic nucleus of these quaternary compounds. Although each of the products of the group showed definite germicidal activity, the introduction of the methyl group was not accompanied by any significant increase in activity. The authors reported that a 1:50,000 aqueous solution of cetylpyridinium chloride killed *S. aureus* in ten minutes at 37° C. but not in five minutes when tested by the F. D. A. method.

Cetyltrimethylammonium bromide which is a compound closely related to cetylpyridinium chloride was reported by Shelton, *et al.* (13), and Barnes (29) to be an excellent disinfecting and cleansing agent. It was able to materially reduce the number of bacteria on normal skin and to completely sterilize dirty bowls. A one per cent aqueous solution was neutral and felt soapy to the touch but was not painful when applied to abrasions of the skin.

Reed, Blubaugh and Gerwe (30) reported on the germicidal activity of cetylpyridinium chloride on various organisms at 20° C. by the F. D. A. method and compared the results with previous findings on the same group of organisms at 37° C. Variation in results at the two temperatures were apparently not due to pathogenicity, morphology or resistance to phenol. Temperature curves were established for four types of germicides: a mercurial, a quaternary ammonium salt, a halogen and a phenol.

Blubaugh, *et al.* (31), used the F. D. A. technique in testing aqueous cetylpyridinium chloride in the presence of serum and concluded that this germicide was highly bactericidal for several species of pathogenic and nonpathogenic organisms. The optimum *p*H for cetylpyridinium was in the range of 6 to 8 while the average *p*H of the aqueous solution is 7.3.

Williams, Clayton-Cooper, Duncan and

Miles (32) recently investigated the cleansing and sterilizing properties of a one per cent aqueous solution of cetyltrimethylammonium bromide. It has considerable antiseptic power when applied to wounds and the skin surrounding wounds. It cleanses and sterilizes instruments. It is also highly effective in removing dirt and bacteria from the hands and is an admirable solution to apply to the hands of surgeons in gloveless surgery. Skin reactions occurred in a small number of cases and it was found that the chemically related determinium chloride is now used exclusively for this purpose. The author concluded that, "It is highly germicidal, has a decided low surface tension action and is nonirritating in the concentration used."

## EXPERIMENTAL

The method used to evaluate the oral antiseptic tested was the method of Hanke (1) and it is quoted here directly from his work.

**Collection of Control Specimen.**—"A piece of sweetened gum is chewed and all of the liquid that collects in the mouth (including the sweet gum extract) is expectorated into a sterile 30-cc. screw-cap jar, until about 25 cc. has been collected. The mixed specimen is divided into two approximately equal parts, one-half to be incubated for four hours. The other half is treated with a fast-acting germicide, such as para-chlormetaxylenol, and set aside in a refrigerator."

**Use of the Oral Antiseptic.**—"From 5 to 10 cc. of the oral antiseptic, the effectiveness of which is to be tested, is introduced into the mouth and retained there, with constant agitation, for two minutes. The liquid is expectorated, but the mouth is not rinsed. Saliva is collected in a sterile jar one, two, four or nine hours after the oral antiseptic has been used. This 25 cc. is also divided into two approximately equal parts. One-half is incubated for four hours and the other half is sterilized as before and placed in a refrigerator."

**Titration of the Samples, No Antiseptic Used.**—"The *p*H of the incubated saliva is determined: exactly 10 cc. of the sterilized portion of the sample measured into the glass potentiometer cup, and the *p*H determined with a Beckman *p*H meter. A 0.1 *N*

TABLE I.—BACTERICIDAL EFFECTIVENESS OF 0.025% CETYLPYRIDINIUM CHLORIDE IN A SOLUTION OF 18% ALCOHOL, 10% GLYCERIN AND DISTILLED WATER

| | No Treatment | | | Three Hours after Treatment | | | |
|---|---|---|---|---|---|---|---|
| Patient | Before Incubation, *p*H | After Incubation for 4 Hr., *p*H | Acid Produced by Bacteria, 0.1 *N* HCl, Cc. | Before Incubation, *p*H | After Incubation for 4 Hr., *p*H | Acid Produced by Bacteria, 0.1 *N* HCl, Cc. | Bactericidal Effectiveness, %[a] |
| 1. T. B. | 6.60 | 5.00 | 1.3 | 7.40 | 7.15 | 0.2 | 84.5 |
| 2. H. L. | 5.75 | 4.85 | 0.5 | 7.15 | 7.19 | 0.0 | 100.0 |
| 3. D. M. | 7.00 | 5.40 | 1.1 | 7.25 | 6.85 | 0.3 | 72.5 |
| 4. M. W. | 5.90 | 4.80 | 0.8 | 7.00 | 6.95 | 0.01 | 98.5 |
| 5. A. C. | 6.60 | 4.70 | 0.8 | 7.50 | 7.30 | 0.06 | 92.5 |
| 6. A. C. | 6.30 | 5.10 | 0.6 | 7.40 | 7.30 | 0.05 | 91.5 |
| 7. M. VC. | 7.80 | 7.35 | 0.5 | 7.45 | 7.55 | 0.0 | 100.0 |
| 8. G. P. | 6.95 | 6.90 | 0.03 | 7.35 | 7.30 | 0.0 | 100.0 |
| 9. C. T. | 4.65 | 4.50 | 0.10 | 6.80 | 6.95 | 0.0 | 100.0 |
| 10. H. R. | 6.20 | 4.75 | 0.8 | 7.35 | 7.45 | 0.0 | 100.0 |
| 11. H. M. | 6.75 | 6.40 | 0.2 | 6.90 | 7.00 | 0.0 | 100.0 |
| 12. R. K. | 6.00 | 4.75 | 0.7 | 6.60 | 6.60 | 0.0 | 100.0 |
| 13. E. A. G. | 6.25 | 4.65 | 0.7 | 6.65 | 6.65 | 0.0 | 100.0 |
| 14. L. M. | 6.45 | 5.75 | 0.4 | 6.65 | 6.85 | 0.1 | 75.0 |
| 15. B. H. | 6.80 | 5.50 | 0.75 | 7.85 | 7.50 | 0.1 | 86.6 |
| 16. M. B. | 5.15 | 5.05 | 0.1 | 6.75 | 6.70 | 0.01 | 90.0 |
| 17. D. S. | 6.30 | 6.10 | 0.15 | 7.00 | 6.90 | 0.1 | 33.5 |
| 18. J. G. | 5.60 | 4.80 | 0.4 | 6.90 | 6.85 | 0.05 | 87.5 |
| 19. C. A. | 6.75 | 5.00 | 1.1 | 7.35 | 7.30 | 0.0 | 100.0 |
| 20. C. H. | 6.75 | 6.00 | 0.7 | 7.10 | 7.10 | 0.0 | 100.0 |
| 21. M. G. | 7.00 | 6.45 | 0.6 | 6.90 | 6.80 | 0.05 | 91.5 |

[a] Average bactericidal efficiency, 90.64%.

gent cetylpyridinium bromide was less likely to induce these reactions.

Auerbach (33) has developed a quantitative method of analyzing dilute solutions of cetylpyridinium chloride.

Powney (34) recently reported that a reaction takes place when sodium hexametaphosphate is added to a solution of cetylpyridinium bromide.

Gledhill (35) has explained how to purify cetyltrimethylammonium bromide.

In a recent study of bronchial lavage in nontuberculous infections, Stitt (36) used 1:5000 cetylpyridinium chloride diluted with Bledsoe–Fisher hypertonic saline solution. Since the results with this solution were better than the results obtained with other well-known germicides, cetylpyri-

hydrochloric acid solution is added in 0.1-cc. portions until the *p*H is identical with that of the incubated portion of saliva. The resulting figure is a direct index of the ability of the bacteria to produce acid in the given sample of saliva."

**Titration of the Samples after Using the Oral Antiseptic.**—"The procedure is exactly as described above, using the specimen obtained after the mouth was rinsed with the oral antiseptic."

**Designation of Results.**—"Bacteriostatic (or bactericidal) effectiveness is conveniently expressed in percentage. Assume, for example, that 10 cc. of a normal sample of saliva, when incubated for four hours, becomes sufficiently acid to reduce the *p*H to 5 and that it has been shown further that 1.0 cc. of 0.1 *N* hydrochloric acid solution has to be added to the sterilized portion of the saliva to reduce the *p*H to 5.0. The oral bacteria in this case produced an acidity equal to that of 1.0 cc. of a 0.1 normal hydrochloric acid solution.

"The incubated portion of the saliva specimen collected from the same person four hours after rinsing of the mouth with a 1:5000 solution of phenyl mercuric nitrate had a *p*H of 6.5. The sterilized portion of this sample had a *p*H of 7 and the addition of 0.1 cc. of 0.1 *N* solution reduced the *p*H to 6.5. The bacteria in the saliva collected four hours after rinsing of the mouth with this phenylmercuric nitrate solution produced only one-tenth as much acid as in the specimen taken just prior to the use of the mouth wash. The bactericidal efficiency is therefore 90% for four hours."

An alcohol, glycerin and water solution containing 0.025% (1:4000) cetylpyridinium chloride was tested by the method of Hanke (1) as previously described. The results are summarized in Table I.

## DISCUSSION OF RESULTS

By comparing the *p*H of saliva after incubation with the *p*H of saliva collected at a definite time after using the germicide, Hanke (1) calculated the effectiveness of various germicides on a percentage basis. In an unspecified number of cases, the bactericidal effectiveness of the various germicides tested was as follows:

| | Time, Hr. | % Effectiveness |
|---|---|---|
| Antiseptic Solution, N. F. | 1 | 45 |
| 3% Hydrogen Peroxide Soln. | 2 | 21 |
| 0.02% Phenyl Mercuric Nitrate | 9 | 56.5 |
| 0.2% Anhydride of 4-nitro-5-hydroxymercuri-*o*-cresol | 2 | 75 |
| 0.02% Sodium Ethylmercurithiosalicylate | 2 | 50 |
| 0.02% Sodium *p*-hydroxymercuri benzoate | 4 | 77.63 |

Cetylpyridinium chloride in 1:4000 concentration in alcohol, glycerin and water solution is bacteriostatic or bactericidal to bacteria found in the oral cavity. This observation is in general agreement with the results of Miller, Baker and Harrison (14) and Miller, Muntz and Bradel (18) on the inhibition of the metabolism of microörganisms found in the mouth by a quaternary ammonium type germicidal agent.

In view of the present state of knowledge of dental caries, no definite conclusions concerning the prevention or treatment of dental caries can be drawn from this work. The efficacy of this agent for the cure, prevention or mitigation of dental caries must be studied clinically on a group with carious lesions and another group without carious lesions. A comparison of the results of the two groups should then throw some light on the efficiency of this compound in the treatment of dental caries.

## SUMMARY AND CONCLUSIONS

1. A review of the quaternary ammonium type germicide cetylpyridinium chloride is presented.

2. Cetylpyridinium chloride in 1:4000 concentration is bacteriostatic or bactericidal to bacteria found in the oral cavity.

3. Using the Hanke method (1) for measuring the bactericidal effectiveness of antiseptics intended to be used in the oral cavity, a solution containing 0.025 per cent (1:4000) cetylpyridinium chloride in a base of 18 per cent alcohol, 10 per cent glycerin and about 70 per cent distilled water has a bactericidal efficiency of 90.64 per cent in an average of 21 cases after a period of three hours.

4. The effectiveness of 1:4000 cetylpyridinium chloride in a base of 18 per cent alcohol, 10 per cent glycerin and about 70 per cent distilled water in inhibiting the bacterial growth of the oral cavity after three hours is greater than the effectiveness reported by Hanke (1) for the National Formulary Antiseptic solution after one hour, 3 per cent hydrogen peroxide after two hours and two well-known organic mercurial germicides after two hours.

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# A Study of Domestic Ergot of Wheat and Rye*,†

*By Arthur E. Schwarting‡ and L. David Hiner§*

The study of *Claviceps purpurea* considered in this study had its inception as a result of the pressure of economic conditions and the lack of literature on the economy and efficiency of production together with fundamental information on the growth habits of this fungus. In this country where most of the cereal hosts of ergot are cultivated, ergot has not assumed a very destructive role. It is only in case of a local epiphytotic that the loss in grain yield caused by ergot becomes an economic factor. Extreme losses due to ergot have never been recorded in the rye crop of the United States but the farmers of the northwest suffered great losses in the durum wheat crop of 1921 due to a heavy infection. Domestic ergot collections make up but a small amount of our needs, and it may be safely estimated that this amount is only a small percentage of the ergot that is lost through a lack of knowledge of an existing market by millers who go to the trouble of separating the ergot and then destroying it.

Agricultural methods are continually improving, both in this country and in Europe. With this improvement, ergot is destined to become more scarce and ultimately artificial methods may have to be relied on to secure adequate quantities of the drug.

The purpose of the investigation follows: (a) To determine the quality of domestic ergots of rye and wheat, and (b) to determine suitable cultural methods for the production of ergot.

## EXPERIMENTAL

The domestic ergots used in the present study include those of various rye and wheat crops of South Dakota, Minnesota, Ohio and those produced experimentally on rye grown in the medicinal plant garden at Ohio State University. The samples used as a basis for this research are included in Table I, which summarizes the numerous research data collected in this investigation.

All of the samples were assayed both chemically and biologically and samples 1, 3 and 4 were used in the field culture studies (see Table I).

**Histology.**—The microscopic morphology of the various rye and wheat ergots was determined by preparing transverse and longitudinal sections of different portions of properly fixed and celloidin imbedded sclerotia. The results of these studies and macroscopic descriptions of the domestic ergots are

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embodied in a thesis which is recorded in the library of Ohio State University.

The pharmacognostical examination of domestic ergot of rye indicates no variation from the U. S. P. description of Ergota except in the measurement of the innermost hyphal cells. The maximum diameter occasionally measured 32 $\mu$ while the U. S. P. gives occasional measurements of individual cells up to 28 $\mu$.

Inasmuch as there is a possibility that ergot of wheat may be recognized therapeutically, it was deemed advisable to start preparation of the monographic material necessary to its adoption by the official books and for commercial identity. The following description is proposed:

### Ergot of Durum Wheat

**Description.—Unground Ergot:** Cylindrical, straight or occasionally slightly curved, the ends blunt, from 5.7 mm. to 18.1 mm. in length and up to 6.4 mm. in thickness, longitudinally furrowed; externally from purple to nearly black; internally white to pinkish white to gray; odor characteristic but free from mustiness or rancidity; taste oily, somewhat acrid.

**Histology:** A thin outer portion of compact hyphal cells generally deep purple to black in color, turning red with 50% sulfuric acid or with chloral hydrate T. S., up to 31 $\mu$ in width and individual cells up to 12.3 $\mu$ in diameter; within this a pseudoparenchyma composed of compact colorless hyphal cells occasionally up to 24 $\mu$ in diameter; slightly elongated and in chains in longitudinal sections and having thin, chitinous walls, and containing protein and numerous globules of fixed oil.

**Powdered Ergot:** Grayish to purplish brown; containing tissue fragments of the outer pseudoparenchyma, colorless hyphal cells in strands and entire, and numerous oil globules.

**Phytochemical Investigation.**—All moisture determinations referred to in this paper were determined by the United States Pharmacopœia XI toluene method (1) (see Table I).

The fixed oil content was determined by packing 10 Gm. of the powdered ergot which had been dried at 100° C. in a Soxhlet extractor. The drug was extracted for ten hours with petroleum ether distilling between 60° and 90° C. The petroleum ether was then evaporated on a steam bath and the residue dried in an oven at 100° C. until a constant weight was obtained.

The chemical assay of the ergot samples was determined according to the method of Hampshire and Page (2). The results of the above methods are shown in Table I. The figures represent the averages of three assays. Comparative results of the alkaloidal content as determined by Hampshire and Page of various Russian and Spanish rye ergots are included in the table for convenience of comparison.

**Pharmacological Investigation.**—The principal motive of the pharmacological investigation was to determine the quality of the domestic ergot samples by the United States Pharmacopœia XI cock's comb method (3). For this purpose fluidextracts were prepared according to the directions of the United States Pharmacopœia XI. Eight select white leghorn roosters were used and the assays were conducted over a period of twenty-two weeks. The results of the assays are included in Table I.

TABLE I

| Sample and Source | % Moisture[a] | % Fixed Oil | % Total Alkaloid (as Ergotoxine) | % in Terms of U. S. P. Standard |
|---|---|---|---|---|
| 1. *S. cerale* L., Minnesota | 3.3 | 29.4 | 0.140 | 110 |
| 2. *S. cerale* L., South Dakota | 6.7 | 24.3 | 0.233 | 140 |
| 3. *S. cerale* L., Minnesota | 4.8 | 25.2 | 0.218 | 110 |
| 4. *S. cerale* L., Ohio | 4.4 | 26.8 | 0.220 | 110 |
| 5. *S. cerale* L., Artificially Produced in Ohio | 5.8 | 21.1 | 0.301 | 130 |
| 6. *T. durum* Desf., South Dakota | 4.1 | 25.9 | 0.131 | 100 |
| 7. *T. durum* Desf., South Dakota | 4.6 | 27.0 | 0.180 | 110 |
| 8. *T. durum* Desf., Minnesota | 5.6 | 27.3 | 0.177 | 100 |
| Russian Rye Ergot | ... | .... | 0.060 | ... |
| Spanish Rye Ergot | ... | .... | 0.223 | ... |
| Spanish Rye Ergot | ... | .... | 0.246 | ... |

[a] U. S. P. Ergota must contain not more than 6% of moisture.

TABLE II

| Condition of Sclerotia | % Germination | Av. No. of Stroma | Date of Germination |
|---|---|---|---|
| One - year - old sclerotia at soil surface Nov. 5 | 83 | 9 | May 10 |
| Two - year - old sclerotia at soil surface Nov. 5 | 47 | 8 | May 10 |
| One - year - old sclerotia in soil Nov. 5 | 92 | 11 | May 10 |
| Two - year - old sclerotia in soil Nov. 5 | 43 | 9 | May 10 |
| Sclerotia in greenhouse at night temperature 45° F. | 26 | 4 | 86 days |
| Sclerotia in greenhouse at night temperature 55° F. | 8 | 5 | 83 days |

## DISCUSSION

Biological and colorimetric methods were included in the present work in an effort to portray a clearer picture of the potency values of the various domestic samples.

The colorimetric method measures total alkaloids in terms of ergotoxine and gives a rough idea of the value of the samples, but does not measure the physiological activity. The cock's comb method, while not measuring the therapeutic value of the sample, gives a measure of the active principles in the drug.

Colorimetric assay results indicate that the domestic samples used are equal to and in most cases show greater potency values than the comparative Russian and Spanish samples. Ergot of rye is higher in total alkaloidal content than the domestic ergot of wheat. In terms of the U. S. P. standard, all of the samples assayed were equal to or of higher potency than the minimum requirement.

Fig. 1.—Germinating sclerotia in soil.

## THE CULTURE OF ERGOT

The culture of ergot is a problem which is not new. All of the results obtained within the last twenty-five years, and dealing both with the culture of *C. purpurea* on artificial media and attempted infections of healthy rye crops have not as yet given a practical solution to the cultivation of this fungus. Much has been written concerning the culture of *Claviceps* on artificial media and conditions for optimum growth and development of the fungus are recorded, but the fungus growth thus obtained has not yielded a product comparable to the sclerotia developed on rye. Experiments trying to infect healthy rye heads have been wholly unsuccessful in some cases and only partially successful in others. Hecke's experiments (4) with hybrids of *Secale cerale* gave excellent yields but whether the infection which he recorded was due to spraying methods or to the nearness of a naturally infected field cannot be ascertained. McCrea (5) concluded that artificial methods are too costly and that weather conditions in Michigan were not conducive to such methods. With these questionable results and conclusions at hand, the present study was undertaken.

It was proposed first to study the factors affecting the germination of the sclerotia, second, to study the natural infection taking place on rye and other grasses and in this way determine the exact nature of the development of the fungus, and third, with this completed, to produce samples of infection on rye by various means and in this way specify the conditions and methods of infection most successful for artificial infection.

## EXPERIMENTAL

In the normal life cycle of the fungus, the sclerotia either fall to the ground before or at the harvest, or are harvested with the grain and then may be planted in the soil with the grain at the time the seed is sown. All of these conditions were duplicated in the test plots and in addition forcing methods were studied in the greenhouse.

Sclerotia of the 1941 crop were placed at the soil surface, some were covered lightly with soil and others were sown with the rye in the fall planting with sand. The sand in the flats was kept continually moist by a wick inserted in the bottom of the flat. These flats were kept in the greenhouse at different temperatures, one at a temperature ranging from 55° F. at night to 75° F. during the day, the other at a temperature ranging from 45° F. at night to 75° F. during the day. The results of these experiments are shown in Figs. 1 and 2, and in the germination record of the sclerotia summarized in Table II.

From the above results the following conclusions were drawn (see Figs. 1 and 2):

Fig. 2.—Germinating sclerotia of ergot of rye.

Fig. 3.—Longitudinal section of nearly mature stroma showing perithecia containing asci. In several perithecia the asci have ruptured and the ascospores may be seen emerging to the surface of the capitulum. ×30.

Fig. 4.—Sphacelial stage showing conidia. ×1000.

of that year. Sclerotia of the 1940 crop were likewise treated in order to study the effects of age on the germination of the sclerotia. Sclerotia which had been kept at room temperature following their collection were sown in flats and lightly covered

1. The power of germination lasts for more than one year but the percentage germination was lower in older sclerotia.
2. Sclerotia forced in the greenhouse do not produce as many nor as large stromata as those which germinate in the normal way.

**Ascospore Dissemination.**—It has been shown that upon rupture of the asci, the ascospores are forcibly ejected into the air and are then readily car-

ried by convection currents to the flowers. In the attempt to isolate ascospores for artificial culture, daily studies were made in the field in the development of the stroma (see Fig. 3). In many cases the ascospores only emerged into the ostiole and ascospores were found on the outer surface of the stroma. It is quite likely that insects which are in abundance at this time of the year are very likely to rub against the stromatal heads, which are covered with ascospores, and subsequently carry them to the rye flowers to increase the infection.

**The Sphacelial Stage.**—The ascospores attack the pistil of the rye flower and in a few days the ovary is almost entirely destroyed, a grayish white mycelial mass occupying the space between the glumes of the rye flower. As this tissue grows and develops, a sticky, sweet exudation is formed by the host and it is in this mass that the conidia are budded off and float to the surface (see Fig. 4). The honeydew stage persists for several days depending on the weather, humid and cloudy days favoring a prolongation of this stage. Conidia do not germinate in the honeydew in an undiluted condition and only following dilution by rain or from some other source such as dew, do the conidia produce germ tubes which are infective.

Fig. 5.—Induced infection of rye by *C. purpurea*. As many as twelve sclerotia appear on single heads.

Fig. 6.—Natural infection of rye by *C. purpurea*. From one to four sclerotia appear on single heads.

## DISCUSSION

Ergot produced purely localized infection, attacking only single florets. There was a definite relationship between the amount of infection to the climate, the infection being favored by wet weather and a reduced amount of sunshine. Conditions such as these appeared favorable for the germination of the sclerotia and for the production and dissemination of the ascospores. During dry weather the flowering takes place rapidly, and the liability of infection is much reduced. The honeydew stage in 1942 was accompanied by much rain but that of 1943 was dry and hot.

### The Field Culture of Ergot

Artificial infections in a plot of rye were first produced in 1942. A rye plot was inoculated with ascospores obtained from germinating sclerotia and were spread to the rye plants by brushing the stroma with a camel's-hair brush and then applying the brush to the flowering head. This process was carried out in early morning over a period of four days. The infection thus produced is shown in Table III and is represented by Fig. 5.

In comparison the table also illustrates the natural infection in a plot where the sclerotia were sown with the grain in the November fifth planting, simulating as nearly as possible the natural field conditions (see Fig. 6).

Ascospores were collected from mature stroma in May of 1942 and were maintained on malt-agar plates until the spring of 1943. Constant biweekly transfers were maintained to insure proper growth.

During that time various liquid media were studied and compared for maximum growth of the fungus. A modified broth, similar to that used by McCrea (6), favored the best development of the fungus and with numerous conidia. The formula follows:

| | |
|---|---|
| Magnesium Sulfate | 1.25 Gm. |
| Dihydrogen Potassium Phosphate | 2.5 Gm. |
| Peptone | 1.25 Gm. |
| Maltose | 12.5 Gm. |
| Yeast Extract | 12.5 Gm. |
| Water | 1000.0 cc. |

TABLE III.—A COMPARISON OF YIELD OF ERGOT BY NATURAL AND ARTIFICIAL METHODS

| Type of Infection | Av. No. of Sclerotia per Head | Weight of Ergot from 100 Infected Rye Heads |
|---|---|---|
| Natural | 1.3 | 10.2 Gm. |
| Artificial | 4.7 | 44.3 Gm. |

A week previous to the contemplated inoculation, a culture of the fungus was started in this broth. The rye crop had been planted on November 12 and the flowers appeared ready for artificial inoculation on June 13. This date was much later than the previous year and can be accounted for by the unusually late spring. On three consecutive mornings a plot of rye, 37 by 24 feet was sprayed with a broth culture containing mycelial masses and conidia. The original broth was diluted one-half with distilled water. The weather during this time was extremely dry and hot, but the infection was quite uniform among the rye plants. Sclerotia collected from this $^1/_{80}$-acre plot numbered 4300 and yielded 4320 Gm. of ergot.

## SUMMARY AND CONCLUSIONS

Experimentation was carried out to analyze various domestic ergots of wheat and rye. Comparative pharmacognostic and pharmacologic studies were made and recorded. Small scale field culture methods were determined for two seasons to ascertain the probable commercial possibilities of the artificial inoculation of rye heads to increase the yield of ergot.

Data collected from a systematic investigation of ergot from 1940 to 1943 established the following conclusions:

1. Structural variations between domestic rye and wheat ergot are limited to compactness of the pseudoparenchyma. Ergot of rye is more deeply fissured than ergot of wheat.

2. Chemical assay methods indicate that domestic ergot of rye and wheat compare favorably with Spanish and Russian samples and that domestic ergot of rye is slightly higher in alkaloidal content than ergot of wheat.

3. Biological assay methods indicate that domestic ergot of rye and wheat meet official standards. Ergot of rye exhibits a higher potency than ergot of wheat.

4. The prevalent climatic conditions of central Ohio favor natural infection of rye, wheat and other grasses.

5. Field culture methods yield increases in infection and yield of ergot of rye. The practical utilization of such methods can only be determined by large-scale operations over a period of years and with suggested controls.

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## WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Adenosine-5-pyrophosphate
Beta amyrin
Bilobol
Cytosine
Thymine
5,6-Cyclopenteno-1,2-benzanthracene
2-Chloromandelic acid
Cyclogallipharic acid
Cholesteryl oleate, palmitate or stearate
Laccol
17-Methyl testosterone
Uricase
2,6,7-Trihydroxy-9-methyl-3-isoxanthone
Formonitrolic acid
Gulonic acid
Mannonic acid
Cerasin
Stilbamidine
Afridol
Promin
3,5-Diiodo thyronine methyl ester
Phenanthridine

# Factors Affecting the *in vitro* Activity of Sulfonamide Ointments*

*By Darwin F. Aldrich† and H. George DeKay‡*

Although the therapeutic possibilities of sulfanilamide had been well established as early as 1932 by the efforts of Domagk and others, it was not until 1939 that any of the sulfonamide drugs were made into ointment form for clinical trial. Since that time the use of ointments as a mode of application of the widely used sulfa drugs has become an accepted practice. The value of these ointments in treatment of many infectious skin diseases has been demonstrated by a large number of workers in the dermatological and medical fields.

The sulfonamides have been incorporated into various kinds of ointment bases. Emulsion bases are preferred by the majority of workers since the pure hydrocarbon bases such as petrolatum do not permit direct contact of the drug with the site of infection and the sulfonamide is not readily liberated from the base. An oil-in-water type of emulsion seems to possess advantages over the water-in-oil type for most purposes since it has a low surface tension allowing more even contact of the drug with the site of infection. A water-in-oil emulsion is more greasy and does not wash off so readily as does the oil-in-water type. These factors have been noted by Strakosch and Clark (1), Shaffer (2), O'Brien and Bonisteel (3) and Miller (4).

Strakosch and Olsen (5) reported that sulfathiazole has decided bacteriostatic action in ointment form on both streptococci and staphylococci. The authors state that "there is a twofold advantage of topical administration of the sulfonamides as compared with their oral administration. First, it can be assumed that the local tissue concentration of the drug will be reached with smaller amounts when the drug is applied locally. Second, the possibility of unpleasant toxic reactions is minimized." Their results indicate that Aquaphor[1] possesses advantages over other bases, and that the maximum bacteriostatic effect of sulfathiazole on cultures of *staphylococcus aureus* is obtained by a 5 per cent concentration.

Favorable results with the use of sulfonamides in ointment form have been reported by many workers, among them being Winer and Strakosch (6), Sams and Capland (7), Keeney (8) and Sheehan and Ferguson (9).

Colebrook in 1941 (10) reported that the local application of 20% sulfanilamide paste containing cod-liver oil is often remarkably effective in clearing up impetigo and allied exudative skin conditions.

In addition to the type of emulsion and the ingredients used in preparing the base for sulfonamide ointments, other factors which play important roles include the *p*H value and the water content. An interesting discussion on the ionization of sulfonamides has been given by Rose (11). The *p*H of the normal intact skin has been determined by several investigators.

Rothman (12), in a review of the principles of percutaneous absorption, has stated that "the problem of determining the composition of an ointment which would attain maximum absorption of incorporated substances is still a problem. No general rule has been established except for the requirement of suppleness." The purpose of this research is to attempt to shed some light on the problem of just what factors are necessary to produce sulfonamide ointments which will attain maximum effectiveness.

## EXPERIMENTAL

Preliminary experimentation with sulfathiazole in a variety of bases was conducted to determine the most effective type of base. Each ointment was tested upon the *S. aureus* organism to determine its relative bacteriostatic power.

**Procedure.**—Sterile meat extract agar was melted and poured into sterile Petri dishes, 15 cc. of agar being added to each dish. Into each dish was pipetted carefully 0.1 cc. of a suspension of the organism from a twenty-four-hour broth culture. After the inoculated agar had hardened, the ointment was enclosed within a glass ring 1.75 cm. in diameter and 4 mm. in height and placed as nearly in the center of the dish as possible. The ointments were introduced onto the agar in such a manner as to insure complete contact of the ointment with the surface of the agar. Triplicate tests were run in all cases. The Petri dishes were then incubated at 37° C. for a period of twelve hours, after which time the dishes were examined to determine the width of the zone of inhibition of growth produced.

Of the ointments tested, the ointment showing

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[1] Obtained from Duke Laboratories, Inc.

the greatest degree of bacteriostatic power was contained in an emulsion base with the following formula:

| | |
|---|---|
| Glyceryl monostearate | 6.3 Gm. |
| Liquid petrolatum | 3.2 cc. |
| Lanolin | 12.6 Gm. |
| Water, *q. s.* to make | 60.0 Gm. |

(NOTE: This base shall hereinafter be referred to as base No. 14.)

Using this base, experiments were next conducted to determine which of the sulfonamides were effective against the staphylococcus organism. It was shown that of the seven drugs tested, sodium sulfathiazole, sulfathiazole and sulfanilamide possessed the greatest inhibitory power, with sodium sulfathiazole having the greatest activity. Sulfadiazine and sodium sulfapyridine were fairly effective, and sulfaguanidine and succinylsulfathiazole did not possess bacteriostatic power to any marked degree.

Further experimentation in which the percentage of the drugs was varied from 1.0% to 10.0% provided results confirming those obtained by previous investigators that maximum effectiveness is obtained with concentrations of the drug from 5.0% to 7.5%.

**The Role of *p*H in Sulfonamide Ointments.**—Ointments of sulfathiazole, sodium sulfathiazole, sulfanilamide, sulfadiazine and sodium sulfapyridine were prepared in which the *p*H values were varied from 4.30 to 11.65, depending upon the drug employed. For the purpose of varying the *p*H values, 1.0% solutions of sodium hydroxide and hydrochloric acid were employed. The *p*H values were determined by means of the electropotentiometer. Base No. 14 was employed throughout the experiment. Five per cent ointments were used in all cases. The ointments were tested upon the staphylococcus in the manner previously described. Table I lists the results obtained.

TABLE I.—RELATIONSHIP OF *p*H VALUES TO BACTERIOSTATIC ACTIVITY

| Sulfonamide | *p*H | Zone[a] |
|---|---|---|
| Sulfathiazole | 4.50 | 34 |
| Sulfathiazole | 6.00 | 34 |
| Sulfathiazole | 8.65 | 38 |
| Sulfathiazole | 9.20 | 40 |
| Sodium sulfathiazole | 4.30 | 34 |
| Sodium sulfathiazole | 5.90 | 35 |
| Sodium sulfathiazole | 8.60 | 38 |
| Sodium sulfathiazole | 10.00 | 43 |
| Sodium sulfathiazole | 11.65 | 38 |
| Sulfanilamide | 4.55 | 33 |
| Sulfanilamide | 7.50 | 33 |
| Sulfanilamide | 8.75 | 38 |
| Sulfanilamide | 10.15 | 31 |
| Sulfadiazine | 4.40 | 28 |
| Sulfadiazine | 7.45 | 33 |
| Sulfadiazine | 9.20 | 41 |
| Sodium sulfapyridine | 5.90 | 25 |
| Sodium sulfapyridine | 9.00 | 26 |
| Sodium sulfapyridine | 10.90 | 43 |
| None (control) | 4.75 | 00 |
| None (control) | 8.20 | 00 |
| None (control) | 11.55 | 00 |

[a] Zone of inhibition, millimeters (av. of 3 or more plates).

**The Role of Water Content in Sulfonamide Ointments.**—Ointments of sulfathiazole and sodium sulfathiazole in 5% strength were prepared in a variety of bases. The water contents were varied from no water up to 75%, depending upon the ability of the base to hold water. The ointments were tested upon the staphylococcus organism.

Table II lists the ointments prepared and the zone of inhibition produced in each case.

TABLE II.—RELATIONSHIP OF WATER CONTENT TO BACTERIOSTATIC ACTIVITY

| Base | Water Content, % | Zone[a] A[b] | Zone[a] B[c] |
|---|---|---|---|
| Vanishing cream | 66 | 40 | 41 |
| Cold cream | 21 | 27 | 36 |
| Cold cream | 50 | 43 | 43 |
| Lanolin | 25 | 29 | 33 |
| Lanolin | 60 | 31 | 40 |
| Aquaphor | 25 | 22 | 31 |
| Aquaphor | 75 | 30 | 42 |
| Hydrosorb[d] | 00 | 30 | 33 |
| Hydrosorb | 50 | 33 | 35 |
| Hydrosorb | 75 | 33 | 38 |
| Simple ointment | 00 | 31 | 30 |
| Simple ointment | 25 | 29 | 31 |
| Cholesterol base | 25 | 27 | 34 |
| Cholesterol base | 50 | 29 | 38 |
| Mucilage tragacanth | 75 | 38 | 43 |

[a] Zone of inhibition, millimeters (av. of 3 or more plates).
[b] A = Sulfathiazole.
[c] B = Sodium sulfathiazole.
[d] Obtained from Abbott Laboratories.

**The Role of Surface-Active Agents in Sulfonamide Ointments.**—Ointments of sulfanilamide, sulfathiazole and sodium sulfathiazole were prepared in the emulsion base No. 14 described previously. In addition, the surface tension reductants Aerosol OT[2] and Duponol WA[3] were added to each ointment in the strengths of 0.5% and 1.0%. These ointments were then tested against the *S. aureus* organism. Table III shows the results obtained.

TABLE III.—RELATIONSHIP OF SURFACE-ACTIVE AGENTS TO BACTERIOSTATIC ACTIVITY

| Sulfonamide | Surface-Active Agent, % | Zone[a] |
|---|---|---|
| Sulfanilamide | A,[b] 0.5 | 40 |
| Sulfanilamide | A, 1.0 | 43 |
| Sulfanilamide | D,[c] 0.5 | 41 |
| Sulfanilamide | D, 1.0 | 40 |
| Sulfanilamide | None | 38 |
| Sulfathiazole | A, 0.5 | 39 |
| Sulfathiazole | A, 1.0 | 43 |
| Sulfathiazole | D, 0.5 | 43 |
| Sulfathiazole | D, 1.0 | 40 |
| Sulfathiazole | None | 39 |
| Sodium sulfathiazole | A, 0.5 | 45 |
| Sodium sulfathiazole | A, 1.0 | 47 |
| Sodium sulfathiazole | D, 0.5 | 50 |
| Sodium sulfathiazole | D, 1.0 | 47 |
| Sodium sulfathiazole | None | 44 |
| None (control) | A, 1.0 | 00 |
| None (control) | D, 1.0 | 00 |

[a] Zone of inhibition, millimeters (av. of 3 or more plates).
[b] A = Aerosol OT.
[c] D = Duponol WA.

[2] The patented name for dioctyl sodium sulfosuccinate, manufactured by the American Cyanamid and Chemical Corporation.

[3] The trade name for a group of fatty alcohol sulfates, manufactured by the E. I. du Pont de Nemours and Company, Inc.

**Tests upon the Streptococcus Hemolyticus Organism.**—As a conclusion to the research a representative group of the ointments used throughout the experimentation was tested upon the *Streptococcus hemolyticus* organism. A pure, virulent strain of *S. hemolyticus* was secured, and blood agar was prepared, using sterile, defibrinated rabbit blood. Agar plates were poured and the ointments were introduced onto the hardened blood agar. The dishes were incubated at 37° C. for twelve hours. The results obtained with the ointments used are given in Table IV.

TABLE IV.—ACTIVITY OF SULFONAMIDE OINTMENTS UPON *Streptococcus Hemolyticus*

| Sulfonamide | Base | Zone of Inhibition, Mm. (Av. of Plates) |
|---|---|---|
| Sodium sulfapyridine | Base No. 14 | 30 |
| Sulfadiazine | Base No. 14 | 28 |
| Sulfadiazine | Vanishing cream | 40 |
| Sulfadiazine | Cold cream | 20 |
| Sulfanilamide | Base No. 14 | 28 |
| Sulfanilamide | Base No. 14, Duponol 1.0% | 30 |
| Sulfathiazole | Base No. 14 | 34 |
| Sulfathiazole | Vanishing cream | 20 |
| Sulfathiazole | Aquaphor, 75% water | 18 |
| Sulfathiazole | Lanolin, 60% water | 20 |
| Sodium sulfathiazole | Base No. 14 | 31 |
| Sodium sulfathiazole | Base No. 14, Duponol 1.0% | 30 |
| Sodium sulfathiazole | Vanishing cream | 32 |

## SUMMARY AND CONCLUSIONS

1. A number of bases were tested for their ability to act as carriers for the sulfa drugs. An ointment base containing 10 per cent glyceryl monostearate, 5 per cent liquid petrolatum, 20 per cent lanolin and 60 per cent water was found to give the best results.

2. Sulfathiazole, sodium sulfathiazole, sulfanilamide and sulfadiazine were shown to have good bacteriostatic activity upon the *S. aureus* organism, with sodium sulfathiazole showing greatest activity. The optimum concentration of the drug appears to be between 5.0 per cent and 7.5 per cent.

3. Optimum *p*H values for ointments of sulfathiazole, sulfanilamide and sulfadiazine were shown to be around *p*H 9.0. For sodium sulfathiazole, the optimum *p*H value in an ointment was observed to be around 10.0.

4. The presence of water in a sulfa drug ointment seems desirable for greater bacteriostatic activity. A water content of at least 50 per cent is recommended for optimum activity.

5. The addition to sulfonamide ointments of the surface tension reductants Duponol WA and Aerosol OT improves the penetration power as shown by the increase in the zone of inhibition, particularly in the case of sodium sulfathiazole. A concentration of the surface tension reductant of 0.5 per cent was observed to have beneficial effects.

6. Sodium sulfathiazole ointment possesses superior bacteriostatic power upon *S. aureus*, whereas sulfadiazine appears to be the most effective against streptococcic organisms, when tested by the cup-plate method for the zone of inhibition of growth.

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# Toxic Effects of Atabrine Following Intravenous Injection*

*By Klaus Unna*†

Since the margin of safety in the intravenous administration of atabrine is small, the use of this drug by the intravenous route for therapeutic purposes has been discouraged. It has been recommended not to exceed 0.2 Gm. in a single dose (1, 2). Intravenous injections of 0.3 or 0.4 Gm. have been reported to cause vomiting, convulsive seizures, circulatory collapse and psychic disturbances (1, 2, 3), acute toxic manifestations which also have been described following the oral administration of massive doses (4, 5). In laboratory animals, the lethal intravenous dose of atabrine is about 20 to 50 times smaller than the peroral dose (6). Dawson, Gingrich and Hollar (7) have demonstrated in rabbits that the lethal dose depends to a large extent on the rate of injection. Thus, a dose of 7.5 mg. per Kg. with a mortality rate of 75 per cent when injected within thirty seconds failed to cause deaths when injected slowly over a period of 150 seconds.

Cardiac disturbance following the intravenous or intramuscular injection of atabrine into animals has been reported. De Langen and Storm (8) obtained extrasystoles in experiments on monkeys and recommended combining the intravenous injection of atabrine with epinephrine. Motta (9) in experiments with rabbits described ventricular extrasystoles and a prolongation of the atrio-ventricular conduction time.

## EXPERIMENTAL

The following studies carried out on dogs and cats are mainly concerned with the influence of the rate of injection of atabrine and with its effects on the circulatory and respiratory system. In the experiments with anesthetized animals continuous records were taken of the carotid blood pressure, rate of respiration, and heart rate, and electrocardiagrams were taken at frequent intervals. Atabrine dihydrochloride dissolved in saline was injected into the jugular or saphenous vein either by syringe, the duration of the injection being controlled by a stop watch, or by means of an infusion pump ensuring a constant rate of infusion. All animals were maintained on standard laboratory diets.

### Fatal Dose and Rate of Injection

**Experiments on Dogs.**—The data obtained with single injections of atabrine in dogs are summarized in Table I. Intravenous injection of 20 mg. per Kg. of atabrine within one and one-half to two minutes caused death within three to thirteen minutes. Immediately after the injection the dogs collapsed, and their respiration became irregular. Convulsive seizures were observed in all animals. Often the dogs came back on their feet for a few minutes, but showed marked incoordination and appeared to be in a stupor. Respiratory movements became gasping and ceased abruptly while the heart was still beating regularly. When the time for the injection was extended to four minutes, the same dose of 20 mg. per Kg. produced as the only toxic manifestation a staggering, incoordinated gait which subsided after twenty minutes. Smaller doses (12 mg. per Kg.) injected over a period of two minutes were tolerated by all dogs without any signs of toxic effects. Repetition of the same dose at the same rate after two hours likewise failed to cause toxic symptoms. Regardless of two preceding injections of 12 mg. per Kg. each, 20 mg. per Kg. injected over a period of two minutes were required to produce death in these animals (dogs 261, 263 and 259, Table I).

The data obtained with prolonged infusion of atabrine through a cannula inserted under local anesthesia into the saphenous vein are shown in Table II. Infusion at the rate of 1 mg. per Kg. per minute caused death after forty-five minutes. This dog developed convulsive seizures during the last minutes of the infusion. The animal became stuporous and died with the same signs of respiratory failure as were observed following rapid injection of the drug. When the rate of the infusion was decreased to 0.5 mg. per Kg. per minute, a total dose of 50 mg. per Kg. was tolerated without any toxic effects, and 75 mg. per Kg. produced only short lasting stupor and some muscular incoordination at the end of the infusion. A total dose of 100 mg. per Kg. was given at a rate of 0.4 mg. per minute to six dogs. All animals survived the infusion. Four dogs showed increased salivation and two made several attempts to vomit. One dog showed a slight stupor and another was unable to stand on his feet. However, after termination of the infusion they recovered rapidly. Appetite was decreased for several days, and the dogs lost from 1 to 2 Kg. in weight, regaining their initial weight slowly after two to three weeks. A total dose of 140 mg. per Kg. was given to a dog at a rate of 0.35 mg. per Kg. per minute. This dog also survived although it developed respiratory disturbances and became greatly excited at the end of the infusion. The dog was unable to stand and remained in an incoordinated state for about thirty minutes after the infusion was terminated. It refused food for several days.

**Experiments on Cats.**—The influence of different rates of injection was studied in 31 cats anesthetized with Dial[1] (40 mg. per Kg. intraperitoneally). The fatal doses established for the different rates of injection are summarized in Table III.

Doses of 10 or 12 mg. per Kg. were fatal when injected within a period of two minutes. This finding is in close agreement with the lethal dose reported by Hecht (6) in experiments on nonanes-

* Received May 18, 1944, from the Merck Institute for Therapeutic Research, Rahway, N. J.

† Appreciation is expressed to Mr. J. G. Greslin and to Miss G. V. Richards for their valuable technical assistance.

[1] A 20% solution of Dial was obtained from Ciba Pharmaceutical Products through the courtesy of Dr. E. Oppenheimer.

TABLE I.—INTRAVENOUS LETHAL DOSE OF ATABRINE IN DOGS

| Dog | Sex | Body Weight, Kg. | Dose, Mg./Kg. | Injection Time, Sec. | Result | Time Interval between Injections |
|---|---|---|---|---|---|---|
| 93 | M | 14.4 | 12.5 | 120 | Survived | ... |
| 260 | F | 11.9 | 20.0 | 90 | Died | ... |
| 264 | F | 6.0 | 20.0 | 90 | Died | ... |
| 262 | M | 10.1 | 20.0 | 120 | Died | ... |
| 50 | F | 10.9 | 25.0 | 120 | Died | ... |
| 261 | M | 10.8 | 12.0 | 120 | Survived | ... |
| | | ... | 12.0 | 120 | Survived | 2 hours |
| | | ... | 20.0 | 120 | Died | 2 hours |
| 263 | M | 10.2 | 12.0 | 120 | Survived | ... |
| | | ... | 12.0 | 120 | Survived | 2 hours |
| | | ... | 20.0 | 120 | Died | 2 hours |
| 259 | M | 8.4 | 12.0 | 120 | Survived | ... |
| | | ... | 12.0 | 120 | Survived | 2 hours |
| | | ... | 20.0 | 240 | Survived | 2 hours |
| | | ... | 20.0 | 120 | Died | 2 hours |

thetized animals. Immediately after the injection the respiration became irregular and ceased entirely within one to two minutes. At the same time the blood pressure fell abruptly while the heart continued to beat regularly.

When atabrine was given by infusion at a rate of 3, 2 or 1 mg. per Kg. per minute the average lethal dose increased from 10 mg. to 53.3 mg. per Kg. (8 experiments), 67.4 mg. per Kg. (9 experiments) and 111.6 mg. per Kg. (5 experiments), respectively. Although the prolongation of the infusion caused a marked increase in the lethal dose, the signs under which death occurred in the infusion experiments were not significantly different from those observed on rapid injection. In all experiments death was due to a sudden respiratory failure followed by rapid fall in blood pressure.

The intravenous injection of 2 mg. of atropine prior to the infusion of atabrine at a rate of 2 mg. per Kg. per minute in six experiments increased the average lethal dose from 67.4 to 98.3 mg. per Kg. (Table III).

### Effect on Respiration and Circulation

In nonanesthetized rabbits and dogs sublethal doses of atabrine injected rapidly within two minutes occasionally produced a temporary increase in the respiratory rate; the prolonged infusion of atabrine accelerated the respiration markedly in several dogs. In the anesthetized animals (cats) no stimulation of the respiration was observed; the injection of sublethal doses (1, 5 or 7 mg. per Kg) did not

TABLE II.—INFLUENCE OF THE RATE OF INTRAVENOUS INJECTION OF ATABRINE UPON THE FATAL DOSE IN DOGS

| Dog | Sex | Body Weight, Kg. | Dose, Mg./Kg. | Injection Time, Min. | Rate of Infusion, Mg./Kg./1.1 Min. | Result |
|---|---|---|---|---|---|---|
| 90 | F | 17.2 | 40.0 | 45 | 1.0 | Died |
| 248 | M | 10.8 | 50.0 | 112 | 0.5 | Survived |
| 247 | M | 12.0 | 75.0 | 168 | 0.5 | Survived |
| 246 | M | 10.9 | 100.0 | 280 | 0.4 | Survived |
| 104 | F | 11.9 | 100.0 | 280 | 0.4 | Survived |
| 236 | F | 9.0 | 100.0 | 276 | 0.4 | Survived |
| 239 | F | 15.3 | 100.0 | 285 | 0.4 | Survived |
| 240 | M | 11.1 | 100.0 | 284 | 0.4 | Survived |
| 241 | M | 10.7 | 100.0 | 281 | 0.4 | Survived |
| 89 | F | 6.8 | 140.0 | 451 | 0.35 | Survived |

TABLE III.—INFLUENCE OF THE RATE OF INTRAVENOUS INJECTION OF ATABRINE UPON THE FATAL DOSE IN CATS ANESTHETIZED WITH DIAL

| Expt. No. | Sex | Body Weight, Mg. | Rate of Infusion, Mg./Kg./-1.1 Min. | Fatal Dose, Mg./-Kg. |
|---|---|---|---|---|
| 1 | M | 3.65 | [b] | 10.0 |
| 2 | M | 2.3 | [b] | 10.0 |
| 3 | M | 3.5 | [b] | 12.0 |
| | | | Av. = | 10.7 |
| 14 | F | 1.82 | 3.0 | 66.0 |
| 15 | M | 2.64 | 3.0 | 51.0 |
| 16 | M | 2.92 | 3.0 | 57.0 |
| 17 | F | 2.55 | 3.0 | 66.0 |
| 18 | F | 1.98 | 3.0 | 39.0 |
| 19 | F | 2.50 | 3.0 | 39.0 |
| 23 | M | 3.35 | 3.0 | 81.0 |
| 24 | F | 3.20 | 3.0 | 27.0 |
| | | | Av. = | 53.3 |
| 6 | F | 2.15 | 2.0 | 36.0 |
| 7 | M | 2.20 | 2.0 | 89.0 |
| 8 | M | 2.18 | 2.0 | 40.5 |
| 9 | M | 2.82 | 2.0 | 81.0 |
| 12 | F | 2.92 | 2.0 | 49.0 |
| 20 | F | 3.50 | 2.0 | 76.0 |
| 21 | M | 2.45 | 2.0 | 66.0 |
| 22 | M | 3.20 | 2.0 | 52.0 |
| 32 | F | 2.10 | 2.0 | 116.0 |
| | | | Av. = | 67.4 |
| 26+[a] | M | 2.82 | 2.0 | 82.0 |
| 27+ | M | 2.00 | 2.0 | 86.0 |
| 28+ | M | 2.69 | 2.0 | 150.0 |
| 29+ | F | 2.10 | 2.0 | 120.0 |
| 30+ | M | 3.20 | 2.0 | 80.0 |
| 31+ | F | 2.40 | 2.0 | 72.0 |
| | | | Av. = | 98.3 |
| 4 | M | 2.40 | 1.0 | 116.0 |
| 5 | M | 3.30 | 1.0 | 85.0 |
| 10 | M | 3.38 | 1.0 | 186.0 |
| 11 | M | 2.67 | 1.0 | 96.0 |
| 13 | M | 3.28 | 1.0 | 100.0 |
| | | | Av. = | 116.6 |

[a] + Received 2 mg. atropine intravenously prior to the infusion of atabrine.
[b] Injected within two minutes.

affect the respiration, and lethal doses injected either at once or infused over periods extending to three hours caused a sudden and complete failure of the respiration often preceded by a short period of irregular breathing (Figs. 1 and 3).

Sublethal doses of atabrine injected within two minutes had no significant effect upon the blood pressure or the heart rate. Doses of 1, 2 or 4 mg. per Kg. usually caused a slight rise in blood pressure, whereas injections of 5 or 7 mg. per Kg. were followed by a transient fall in blood pressure without any effect on the heart rate. These doses were given repeatedly within a period of one hour without any indication of cumulative effects. The rapid injection of a lethal dose of atabrine caused an abrupt fall in blood pressure coinciding with the arrest of the respiration (Fig. 1). The heart continued to beat for several minutes after the respiratory and circulatory collapse, and electrocardiograms taken during this period showed regular sinus rhythm.

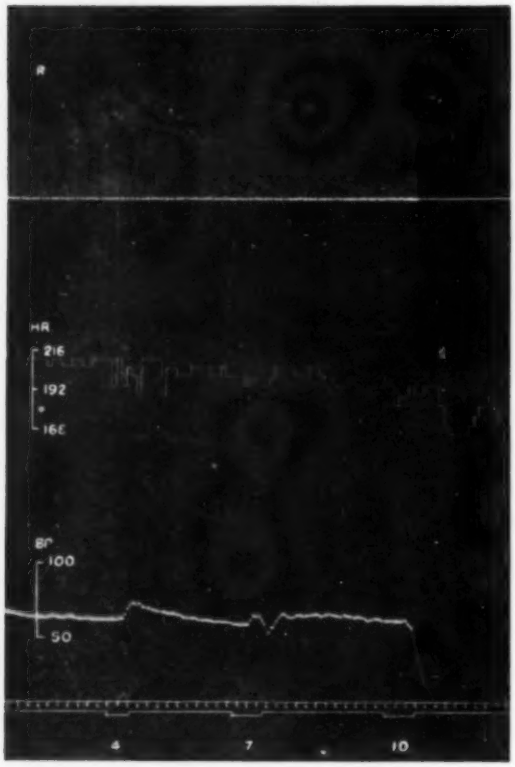


Fig. 1.—Effect of intravenous injection of Atabrine.

Cat 3.1 Kg. Dial anesthesia (40 mg./Kg. intraperitoneally). R = Rate of respiration; H R = Heart rate, beats per min.; B P = carotid lood pressure, mm., Hg. Time intervals 30 sec. The signals indicate the injection of 4, 7 and 10 mg./Kg. of atabrine, respectively.

In the experiments with prolonged infusion of atabrine, there was usually a slight initial increase in blood pressure; later, the blood pressure showed a gradual decline. Comparable to the experiments with rapid injection of the drug, death in the infusion experiments occurred suddenly with the cessation of the respiration and concomitant drop in blood pressure to 0. The heart rate decreased from an average of about 200 beats per minute to about 130. The slowing of the heart rate was accompanied by an increase in the atrioventricular conduction time from an average of about 60 milliseconds to 85 milliseconds. Premedication with atropine failed to prevent the decrease in the heart rate following atabrine in five out of six experiments. The increase in the conduction time was likewise not influenced by atropine (Fig. 2).

### Antagonistic Effect of Picrotoxin

Since intravenous administration of toxic doses of atabrine causes signs of paralysis of the central nervous system, a possible antagonistic effect of picrotoxin was studied on cats and on rabbits.

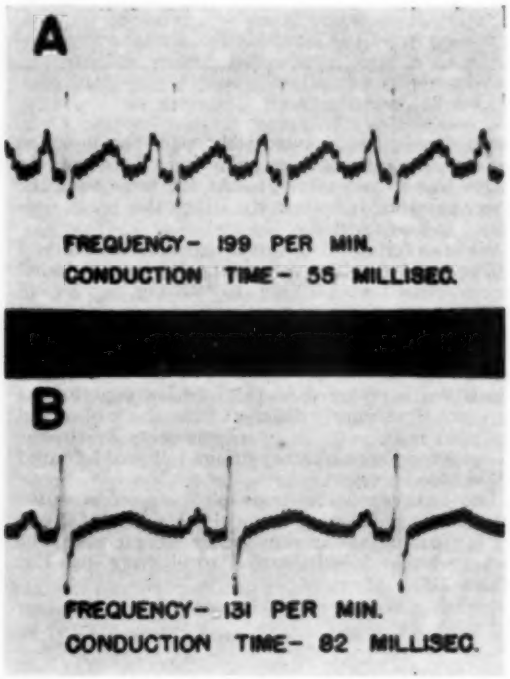


Fig. 2.—Effect of Atabrine on the heart as shown by the electrocardiogram.

Cat 2.4 Kg. Dial anesthesia. 2 mg. atropine intravenously A = before infusion. B = at the end of the infusion of 72 mg. per Kg. of atabrine at a rate of 2 mg./Kg./1.1 min. The respiration had ceased and the blood pressure had fallen to 0.

In 4 cats receiving lethal doses of atabrine by infusion, picrotoxin was injected after the respiration had ceased completely. Figure 3 illustrates a typical experiment. After 40.4 mg. per Kg. of atabrine given at a rate of 3 mg. per Kg. per minute the respiration ceased abruptly. The injection of 0.5 mg. per Kg. of picrotoxin into the common carotid artery one minute later promptly caused a resumption of the respiration and a steep increase in blood pressure. A second injection of picrotoxin given intravenously enhanced the effect of the first. The respiration became markedly accelerated and increased in depth; muscle fibrillation and mild convulsive seizures were observed. The infusion of atabrine resumed at the time of the second injection of picro-

TABLE IV.—EFFECT OF PICROTOXIN ON INTRAVENOUS TOXICITY OF ATABRINE IN RABBITS[a]

| No. of Animals | Atabrine, Mg./Kg. | Picrotoxin, Mg./Kg. | Survived | Died | Time of Death after Injection |
|---|---|---|---|---|---|
| 6 | ... | 0.5 intrav. | 6 | 0 | ... |
| 6 | ... | 1.0 intrav. | 6 | 0 | ... |
| 15 | 8 | ... | 2 | 13 | 3 to 5 min. |
| 5 | 8 | 0.1 intrav. simultaneously | 1 | 4 | 2, 3, 6, 7 min. |
| 10 | 8 | 0.3 intrav. immediately following | 8 | 2 | 3 to 69 min. |
| 18 | 8 | 0.3 intrav. simultaneously | 13 | 5 | 5, 5, 6, 10, 31 min. |
| 6 | 8 | 0.5 intrav. simultaneously | 0 | 6 | 5, 5, 10, 20, 21, 50 min. |
| 10 | 8 | 0.6 subcut. 30 minutes before | 6 | 4 | 3, 4, 4, 5 min. |

[a] All injections of atabrine were made in 90 sec.

toxin was continued for another thirteen minutes until the death of the cat. The lethal dose of atabrine (35.1 mg. per Kg.) after the administration of picrotoxin was almost as large as that of the first infusion (40.5 mg. per Kg.) from which it was separated by an interval of only two minutes.

In this and similar other experiments, picrotoxin not only counteracted the effect of lethal doses of atabrine but also apparently increased the resistance of the animal to the intravenous injection of atabrine. In one experiment, picrotoxin was given twice to prevent the death of the animal, first after 63 mg. of atabrine (2 mg. per Kg. per minute) and again after 44 mg. A third infusion had to be carried to 52 mg. to cause the death of the animal.

The data obtained on rabbits are summarized in Table IV. Simultaneous injection of 0.1 mg. picrotoxin with 8 mg. atabrine had no significant effect on the mortality. However, a reduction in the mortality of atabrine was obtained when 0.3 mg. picrotoxin was injected immediately following or simultaneously with atabrine. Breathing became accelerated, but no irregularities or convulsions were noticed in the animals which survived. The simultaneous injection of 0.5 mg. picrotoxin failed to reduce the fatality of atabrine. The onset of convulsions and of the respiratory disturbances, however, were delayed in the majority of this series; after fifteen minutes the rabbits became restless, developed muscle twitches and marked salivation—signs suggestive rather of picrotoxin than of atabrine poisoning. Picrotoxin alone (0.5 mg.) did not produce toxic manifestations other than acceleration of the respiration, increased excitability and excessive salivation lasting for about one hour; even 1.0 mg., causing marked excitement, salivation and convulsive seizures after twenty minutes, was tolerated. The administration of picrotoxin (0.6 mg.) by subcutaneous injection thirty minutes before, afforded protection against atabrine to a lesser degree than that observed with the simultaneous injection of 0.3 mg. picrotoxin.

The toxicity of 10 mg. of atabrine, which when injected over a period of ninety seconds caused death usually within three minutes or less, was not influenced by the intravenous or subcutaneous administration of picrotoxin.

Metrazol (5 or 10 mg. per Kg.) injected simultaneously with 8 mg. per Kg. atabrine failed to influence the effect of atabrine.

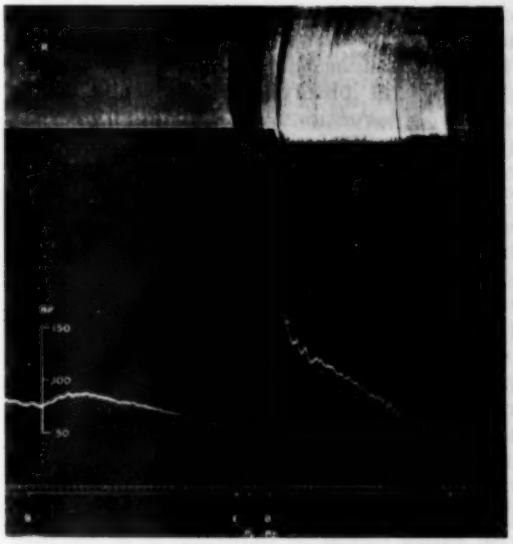


Fig. 3.—Antagonistic Effect of Picrotoxin on Atabrine.

Cat 2.8 Kg. Dial anesthesia (40 mg./Kg. intraperitoneally). R = Rate of respiration; B P = carotid blood pressure, mm. Hg. Time intervals 30 sec. Infusion of atabrine (3 mg./Kg./min.) began at B and ended at E. Picrotoxin (0.5 mg./Kg.) injected into the common carotid artery at $P_1$. The infusion of atabrine (3 mg./Kg./min.) was resumed at B simultaneously with an intravenous injection of picrotoxin (0.5 mg./Kg.) at $P_2$.

## DISCUSSION

The fatal dose of atabrine is largely dependent upon the speed of the injection. In this respect our experiments on dogs and cats confirm the observations made in rabbits by Dawson, Gingrich and Hollar (7). In dogs, doses seven times greater than the lethal dose following rapid injection are tolerated when infused slowly over a period of several hours, and in cats the fatal dose by infusion at a rate of 1 mg. per Kg. per minute (116 mg. per Kg.) is ten times greater than that following rapid injection of the drug (10.7 mg. per Kg.). Hence, the margin of safety in the intravenous administration of atabrine is greatly increased by a slow rate of injection. Furthermore, repeated injections of small amounts which do not cause toxic effects allow the administration of a total dose of atabrine well in excess of the relatively small amount which is fatal when injected at once.

In infusion experiments there was evidence of vagal stimulation. Salivation was observed frequently in dogs. One dog (No. 90, Table II) vomited repeatedly during

the infusion; on autopsy the stomach was found markedly distended containing large amounts of food. In three of the dogs receiving 100 mg. per Kg. attempts to vomit after 80 mg. per Kg. had been administered coincided with the beginning of increased salivation. Vomiting or attempts to vomit were not observed in any of the other 14 dogs, a finding contrasting sharply with the regular occurrence of vomiting upon oral administration of suitable doses of atabrine in dogs.

The decrease in the heart rate observed in cats was apparently not due to vagal stimulation, since atropine did not prevent it. The increase in the atrio-ventricular conduction time observed in cats agrees with similar observations by Motta on rabbits (9). However, contrary to the experience of Motta on rabbits and of De Langen and Storm (8) on monkeys, intravenous administration even of fatal doses did not produce irregularities in the heart beat of cats. As evidenced by the electrocardiogram, the heart was beating in regular sinus rhythm throughout the infusion of atabrine.

Regardless of the rate of the intravenous injection, death was not due to heart failure; rather it appears to result from paralysis of the respiratory and vasomotor centers. In all experiments the respiratory and circulatory collapse occurred suddenly, the blood pressure fell abruptly without the preceding rise which would be expected as a response to asphyxia after the cessation of the respiration.

Picrotoxin when injected immediately into the carotid artery was found to counteract the fatal collapse in anesthetized cats. The blood pressure returned to a normal level and the respiration was resumed almost immediately. In rabbits intravenous or subcutaneous administration of picrotoxin in suitable doses accelerated the respiration and prevented or delayed the respiratory disturbances and convulsions of atabrine; it reduced the mortality of a minimum lethal dose of atabrine (Table IV). However, picrotoxin was ineffective when the dose of atabrine was increased by 20 per cent. It appears, therefore, that the sudden death following intravenous administration of atabrine is due to a flooding of the vital nerve centers when the drug is administered too rapidly to allow for a sufficient absorption from the blood stream by the liver and other organs.

## SUMMARY

1. The margin of safety in the intravenous administration of atabrine is greatly increased by a slower rate of injection or by repeated injections of divided doses. Decreasing the speed of injection from 5 to 1 mg. per Kg. per minute resulted in a tenfold increase of the fatal dose in cats. By slow infusion dogs tolerated an amount of atabrine seven times greater than the dose fatal on rapid injection.

2. Death following intravenous administration of atabrine in cats and dogs is due to paralysis of the respiratory and vasomotor centers. Irregular breathing, stupor and muscular incoordination often precede the terminal collapse.

3. The heart rate decreases during the infusion of atabrine in cats, the conduction time is prolonged, but arrhythmia has not been observed.

4. Atropine does not prevent the bradycardia but it increases the lethal dose of atabrine administered by slow infusion.

5. Picrotoxin counteracts the collapse of the respiration and blood pressure in cats. In rabbits, under certain conditions, it reduces the mortality of atabrine.

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# Anti-Bacterial Properties of Some Mercurials from Aliphatic Glycols*

*By Bernard Heinemann*†

Aromatic derivatives of mercury are widely used clinically as antiseptics. The aliphatic derivatives of mercury, in spite of the fact that many are well known to chemists, have been almost completely neglected in so far as their anti-bacterial properties are concerned. Ethyl mercury chloride (1, 2) has been found to be useful as an agricultural disinfectant for plant pathogens. Bush and Bass (3) investigated a series of simple alkyl mercuric chlorides as "skin-sterilizing" agents.

It has been well established that in a given series of compounds, which can be distributed between water and a lipoid or lipoid solvent, the better anti-bacterial agents are those which distribute themselves in favor of the lipoid or lipoid solvent. A survey of the literature showed that few, if any, such studies have been conducted on mercurials.

Three series of mercurials, a total of fifteen compounds, were prepared in this laboratory from aliphatic glycols by Shukis and Tallman (4). The compounds synthesized were of the following types:

(I). $CH_3—CH_2—(O—CH_2—CH_2)_n—O—CH_2—CH_2—Hg—Cl$

(II). $HO—CH_2—CH_2—(O—CH_2—CH_2)_n—O—CH_2—CH_2—Hg—Cl$.

(III). $HO—(CH_2)_n—O—CH_2—CH_2—Hg—Cl$.

In addition, certain derivatives of these general types were prepared for purposes of comparison.

The purpose of this paper is to report on the anti-bacterial properties of the above three series of mercurials prepared from aliphatic glycols and to compare these properties with the ratio of their solubility in benzene/solubility in water

## EXPERIMENTAL

The methods of preparation of the compounds and determination of their distribution coefficients, $K_D$ (solubility in benzene/solubility in water) have been described (4).

Twenty-four-hour broth cultures of *Staphylococcus aureus* No. 209 were used to determine the bactericidal dilutions. Various dilutions of the mercurials were made in 5-cc. volumes with sterile distilled water or other suitable solvent, as noted in Table I. Lowest dilutions of the compounds tested were determined by their solubility either in water or in a concentration of the solvent used which was not lethal to the test organisms. *Control tests were performed to insure the absence of killing activity by the concentration of solvent used.* One-half cubic centimeter of test culture was pipetted into 5 cc. of each dilution, at 20° C., mixed and allowed to stand at this temperature for ten minutes. At the end of this interval a subculture was made with a 4-mm. loopful (of No. 23 B. and S. gauge wire) to 10 cc. Thioglycollate Medium (Baltimore Biological Laboratories) and incubated at 37° C. for at least forty-eight hours.

Bacteriostatic concentrations were determined by mixing 1 cc. of a twenty-four-hour broth culture of *S. aureus* No. 209 with 100 cc. nutrient broth and adding this to sterile test tubes containing varying amounts of the mercurial until the total volume was 10 cc. The concentration of mercurial which inhibited visible growth for forty-eight hours at 37° C. was taken as the bacteriostatic end point.

Agar-cup plates were prepared according to the procedure given in Circular No. 198 of the U. S. Department of Agriculture. The mercurials for these tests were prepared in 20 p. p. m. concentration in distilled water. Nutrient agar (Difco) was used for the plates and the zones of inhibition were measured after forty-eight hours' incubation at 37° C. The test organism was *S. aureus* No. 209.

## RESULTS

In Table I the melting points $K_D$ values, bacteriostatic concentrations, agar-cup zones and bactericidal dilutions for the compounds employed in the tests are summarized. Increasing the number of ethoxy groups from one to five, in Series I, was accompanied by a stepwise decrease in the solubility coefficient and an increase in the quantity of the mercurial necessary to act as a bacteriostatic agent. Also with the higher members of this series the zones formed around the agar-cups became smaller, indicating a decreased ability to penetrate the agar and inhibit growth. None of the compounds in this series showed evidence of bactericidal activity in the dilutions tested.

The compounds of Series II do not form a closely related series since Nos. 7 and 8 each formed a di-mercurial on synthesis. Both di-mercurials gave higher $K_D$ values than the mono-mercurial of the series and exhibited bactericidal properties. A $K_D$ value of 48.0 was given by compound No. 7

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and it was lethal to *S. aureus* in a dilution of 1:2000 (25% ethanol). Compound No. 8 gave a lower $K_D$ of 15.2 and killed the test organism at a dilution of 1:1000 (25% ethanol). The mono-mercurial of this series, compound No. 6, exhibited a $K_D$ of 3.6 and was not lethal to *S. aureus* in a suitable concentration for testing. The bacteriostatic concentrations and agar-cup zones, for the two di-mercurials in this series, also gave a good correlation with the $K_D$ values obtained for them. The mono-mercurial, compound No. 6, showed greater bacteriostatic activity (1.7 p. p. m.) in spite of a $K_D$ value which was lower than that of the other two di-mercurials of the series.

In Series III compounds Nos. 9, 11, 14 and 15 form a structurally related series. Compound No. 11 showed the highest $K_D$ value of these four compounds and this was accompanied by a bactericidal dilution of 1:2500 (25% ethanol). The degree of antibacterial activity for the other three compounds was correlated with the $K_D$ values obtained for them, as shown in Table I.

The effects produced as the result of placing substituents on the aliphatic chain is worthy of note. Substituting —H, —Cl, and —OH for X in the type compound $XCH_2—CH_2—O—CH_2—CH_2—Hg—Cl$ caused a marked effect on the $K_D$ values obtained and the effectiveness of antibacterial activity. When X is —H (compound No. 1) a $K_D$ of 14.0 was obtained with a bacteriostatic concentration of 0.4 p. p. m. and agar-cup zone of 8.0 mm.; the compound was not bactericidal at a dilution of 1:400. When X is —Cl (compound No. 10) a higher $K_D$ of 29.3 was obtained and this compound was bactericidal to *S. aureus* at a dilution of 1:400, while the results for bacteriostatic concentration and agar-cup zone of inhibition were of nearly the same magnitude as for compound No. 1. When X is —OH (compound No. 9) the $K_D$ dropped to 0.06 and this compound exhibited a bacteriostatic end point at 1.2 p. p. m. and an agar-cup zone of 2.0 mm.; it was not lethal to the test organism at a dilution of 1:200.

A $K_D$ value could not be obtained for compound No. 13, 2,3-dihydroxypropoxy ethyl 2-mercuric chloride, due to its insolubility in benzene. This derivative is known to be quite water soluble and gave a bacteriostatic concentration of 2.0 p. p. m. and an agar-cup zone of 2.0 mm.; it was not lethal to *S. aureus* at a dilution of 1:200. Compound No. 12, 2-hydroxypropoxy ethyl 2-mercuric chloride, had a higher $K_D$ of 3.8 and 0.7 p. p. m. was bacteriostatic to the test organism. This compound was not bactericidal in a dilution of 1:250. 3-Hydroxypropoxy ethyl 2-mercuric chloride,

TABLE I.—PHYSICAL CONSTANTS AND ANTI-BACTERIAL PROPERTIES OF SOME MERCURIALS PREPARED FROM ALIPHATIC GLYCOLS

| No. | $n$ | M. p., ° C. | $K_D$ | Bacteriostatic Concentration, P. P. M. | Agar-cup Zones, Mm. | Bactericidal Dilution and Solvent |
|---|---|---|---|---|---|---|
| | Type I. | $CH_3—CH_2—(O—CH_2—CH_2)_n—O—CH_2—CH_2—Hg—Cl$ | | | | |
| 1 | 0 | 92 | 14.0 | 0.4 | 8.0 | (−)1:400 (20% acetone)[a] |
| 2 | 1 | 34–35 | 7.8 | 0.8 | 6.5 | (−)1:200 (20% acetone) |
| 3 | 2 | 50 | 7.3 | 1.0 | 5.5 | (−)1:200 (water) |
| 4 | 3 | 53–54 | 3.9 | 2.0 | 2.5 | (−)1:200 (water) |
| 5 | 4 | Oil | 2.1 | 6.0 | 0 | (−)1:200 (water) |
| | Type II. | $HO—CH_2—CH_2—(O—CH_2—CH_2)_n—O—CH_2—CH_2—Hg—Cl$ | | | | |
| 6 | 1 | 70–72 | 3.6 | 1.7 | 1.5 | (−)1:450 (25% ethanol) |
| 7 | 2[b] | 90–91 | 48.0 | 2.5 | 4.5 | 1:2000 (25% ethanol) |
| 8 | 3[b] | 86–87 | 15.2 | 3.0 | 3.0 | 1:1000 (25% ethanol) |
| | Type III. | $HO—(CH_2)_n—O—CH_2—CH_2—Hg—Cl$ | | | | |
| 9 | 2 | 88–89 | 0.06 | 1.2 | 2.0 | (−)1:200 (water) |
| 10 | 2[c] | 54 | 29.3 | 0.4 | 9.0 | 1:400 (20% acetone) |
| 11 | 3 | 114–116 | 6.0 | 0.8 | 3.0 | 1:2500 (25% ethanol) |
| 12 | 3[d] | 80–81 | 3.8 | 0.7 | 3.0 | (−)1:250 (water) |
| 13 | 3[e] | 88–91 | 0 | 2.0 | 2.0 | (−)1:200 (water) |
| 14 | 4 | 92–93 | 0.46 | 0.7 | 2.5 | 1:200 (20% acetone) |
| 15 | 6 | 98–99 | 3.7 | 0.4 | 4.0 | 1:500 (25% ethanol) |

[a] (−) indicates that the preparation was not lethal to *S. aureus* at the concentration stated.
[b] Dimercurials with —HgCl on both ends.
[c] Prepared from ethylene chlorohydrin; differs from type formula by —Cl in place of —OH.
[d] Compound prepared from propylene 1,2 glycol and is the isomer of No. 11.
[e] Compound prepared from glycerol and has one more —OH than the type formula. Insoluble in benzene.

compound No. 11, gave a $K_D$ value of 6.0 and killed *S. aureus* in a solution of 1:2500 (25% ethanol).

## DISCUSSION

The correlation between solubility coefficient ($K_D$ value) and antibacterial activity finds good agreement in the series of mercurial compounds studied. It appears that the generalization relating the more active compounds with the more water-insoluble members in a series of structurally related compounds is valid for the mercurials tested. The distribution coefficient of the compounds studied was altered by changing the ratio of —OH and —O— to the $—CH_2$ groups within a molecule. In general, the addition of oxygen-bearing groups to a molecule, as in Series I, lowers the $K_D$ value and decreases anti-bacterial activity. On the other hand, $—CH_2$ groups added to the structurally related compounds of Series III tended to raise the $K_D$ value and increase anti-bacterial activity.

Alteration in the structure of the compounds in a series changes this relationship as is shown in the case of the di-mercurial members of Series II and certain substituted members of Series III.

In Series I the extent of anti-bacterial activities as evidenced by bacteriostatic concentrations and agar-cup zones was clearly shown to be correlated with the magnitude of the $K_D$ value. With the homologous compounds Nos. 9, 11, 14 and 15 of Series III this correlation could be extended to bactericidal activity.

The magnitude of $K_D$ need not be directly proportional to the degree of antibacterial activity in a series of structurally unrelated compounds. An example of this is compound No. 11 which exhibited a $K_D$ value of 6.0 and was lethal to the test organism in a dilution of 1:2500; while compound No. 7 had the highest $K_D$ value, 48.0, of all the mercurials tested but was lethal only in a dilution of 1:2000.

The necessity of using a neutralizing medium, such as Thioglycollate Broth, for subcultures in determining the bactericidal activity of mercury-containing compounds has been discussed previously (5).

## SUMMARY

The ratios of solubility in benzene/solubility in water ($K_D$ value) of three series of mercurials, a total of 15 compounds, prepared from aliphatic glycols were compared with their bacteriostatic concentrations in nutrient broth, agar-cup zones of inhibition and bactericidal dilutions using *S. aureus* No. 209 as the test organism.

In general, the more water-insoluble members of a given series were the better antibacterial agents. The distributon coefficients and thus also the anti-bacterial activity could be changed by altering the ratio of —OH and —O— to the $—CH_2$ groups within a molecule. In the series of mercurials studied the addition of oxygen-bearing groups lowered the $K_D$ value and decreased antibacterial activity while the addition of $—CH_2$ groups tended to raise the $K_D$ value and increased the activity.

The placing of substituents on the aliphatic chain had a marked effect on the $K_D$ value and the anti-bacterial activity.

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# The Effects of Certain Harvesting and Mulching Practices on the Yield and Survival of Belladonnna*,†

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When belladonna is grown as a one-year crop, as was largely the case in 1942 when more than 500 acres were under cultivation in the United States, the grower naturally resorts to practices which will assure the largest returns for that year without thought about the effect of such practices on the survival of the plant after the end of the growing season. As long as the quality of the herb harvested is satisfactory, the question is largely whether repeated harvesting produces greater net returns than one or two harvests. Facilities for drying the crop and the time available for the harvests are also factors which determine the practice followed. On several hundred acres successfully grown in Wisconsin and Pennsylvania in 1942, the crop was cut from one to five times, depending largely on the drying facilities available.

Although production records from many of these growers (1) were secured, no clear indication could be obtained regarding the relation of frequency of cutting to total yields. In Wisconsin where all the growers reporting cut the crop at least two or three times and some of them as often as four and five times the average per acre yield was 937 pounds of dry herb. In Pennsylvania where much of the acreage was cut less frequently the average yield was 716 pounds. However, the highest yield from any individual field, namely 1786 pounds, was reported from Pennsylvania whereas the highest in Wisconsin was 1400 pounds. The question of quality is also involved. When cut frequently the harvested material contains relatively little ripe or even green fruit which is generally present if only one or two cuttings are made and which makes the drying of the herb more difficult. Inasmuch as no two commercial fields even in the same locality are entirely comparable with respect to all conditions exact comparisons cannot be made of yields from such fields under different harvesting procedure. Replicated plot experiments are necessary to determine whether total seasonal yields of dry herb are related to the number of cuttings made.

Much of the acreage in Wisconsin and Pennsylvania was plowed up in the fall but enough was held over to afford some valuable observations on the survival of the plants under winter conditions. With few exceptions the rate of survival was extremely poor, justifying the general conclusion that under the weather conditions that prevailed a second-year crop cannot be obtained unless the practices that were followed are modified.

Failure of the belladonna plant to survive the winters may be due to any one of several conditions or to a combination of these. Extremes of temperature, lack of snow and other conditions not subject to control may be partly responsible. Heavy and poorly drained soils are generally believed to result in greater loss of plants as reported by Laurie (2) and Brewer and Laurie (3) who also found that a heavy mulch of strawy manure is very beneficial on light soil but that a lighter mulch is preferable on heavy, clay soil. The published accounts by these investigators do not indicate the size of the plots or the number of plants on which their conclusions are based. Grove (4) observes that there is evidence in Pennsylvania that the roots overwinter better if the plants have had some time to recover between the last cutting and killing frost. From replicated plot experiments in 1942 the present writers (5) found that without mulching the loss of one-year-old plants was almost complete but that about 50 per cent survived when a 4-inch straw mulch was used while little benefit resulted from covering the plants with soil.

Although the failure of belladonna to overwinter successfully has always been a problem to growers, the early published accounts of belladonna culture in this country are strangely lacking in any reliable data bearing on the conditions that are primarily responsible for the poor survival of this plant. Means of overcoming the difficulty have been resorted to by the successful growers, such as mulching and removing the roots for replanting in the spring but no reports appear to have been published of experiments conducted in sufficient detail and under conditions which make it possible to determine the significance of the data obtained.

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† Credit is due W. E. Domingo for making the statistical analysis of the data.

It was for the purpose of determining the relation of frequency of cutting to the yield and quality of herb obtained and of such frequency and the use of straw mulch to winter survival that a replicated plot experiment was undertaken in 1943 and completed in the spring of 1944. This paper reports the results of that experiment.

## EXPERIMENTAL

The experiment involved the use of 16 plots arranged in 4 blocks of 4 plots each. The plots had four rows 75 feet long, spaced 3 feet apart and each row set with 50 plants spaced 1.5 feet apart. Only the two inner rows were used in recording data, the outer ones serving as border rows. Four harvesting treatments were used, which for convenience are designated as (*A*) not harvested, (*B*) harvested once, (*C*) harvested twice and (*D*) harvested three times. All treatments were replicated four times, making 16 plots in all and 3200 plants, one-half of which furnished the data recorded.

The plantings were made on a medium heavy loam somewhat lacking in adequate drainage in one edge of the field. The plants were grown in the greenhouse and transplanted to the plots May 11, 1943. Quite a number of plants died from various causes early in the season, hence there was not a complete stand in any plot, the total number of plants in the two data rows in each plot ranging from 71 to 96. The border rows were treated the same as the data rows in each case.

The treatment designated as *A* which called for no harvesting of the crop was included to provide a basis of comparison of winter survival with the other plots in which the plants were harvested from one to three times. The dates of the several harvests were so selected that the maximum growth between harvests would result. Thus the plants that were to be harvested three times were cut earlier and later than the others and those that were to be harvested only once were allowed to grow until September 2 to assure maximum yield without too great a proportion of fruit.

All plants were cut 6 to 8 inches above the ground because previous experiments (5) had shown that cutting them close to the ground resulted in severe loss of plants during the season. The cut herb from each replicated plot was weighed, thoroughly mixed and a portion weighing not less than 10 pounds dried in a drying chamber at about 120° F. and ground to provide a representative sample for assaying. The data on yield and alkaloid content resulting from each harvesting treatment are assembled in Table I.

The growing season extended late into the fall and a killing frost did not occur until November 11. The following day a straw mulch was applied on some of the rows in each plot according to a plan which would afford the largest number of comparisons of its effects. The straw, mostly oat straw, was applied loosely 8 to 12 inches deep which settled to about half that thickness after several rains.

The plants under treatment *A* which had not been harvested were about 2 feet tall at this time while those under treatment *B* which were harvested once (September 2) had made new growth from 6 to 12 inches in height. Since the tops in both cases would very likely provide some protection to the plants it was removed in two of the four rows of all the plots to which treatments *A* and *B* were applied and in each case one such row was mulched with straw and the other was left naked. The plants harvested September 15 made only short new top growth and those harvested September 28 made practically no growth. All data relating to survival under the several treatments are shown in Table III.

## RESULTS AND CONCLUSIONS

The yields of dry herb obtained under these several harvesting procedures were quite satisfactory considering that the season was not altogether favorable. The rainfall in late summer and fall was much below normal and this probably reduced the yield from the cuttings made on September 28. In order to bring the relative yields into more easily understood comparison the per acre yields are shown as calculated from the yields obtained from each replicated plot. It will be noted that these plots which were set with 50 plants in each row in no case had the full number of plants when the several cuttings were made. The highest number harvested in any plot (two data rows) was 96 and the lowest 68. While the actual stand of plants in some replications is thus seen to have been considerably below the number that could be grown in the space indicated such conditions often occur in commercial acreages where they may be an important factor in reducing the yield obtained. The calculated yields of 1037, 1270 and 1050 pounds per acre from one, two and three cuttings, respectively, are obtained on that basis.

Due to the lower fertility of the soil at one end of the field where the fourth replications of the several treatments were located the yield from this replication was in all cases the lowest. When two cuttings were made the yield from the second was substantially larger than that from the first in three of the four replications. When three cuttings were made the yield from the first was in all cases much smaller than that from the later ones and that from the last one, made on September 28, yielded the largest amount by a considerable margin.

When the data on yields are analyzed by the variance method it is found that the frequency of cutting the crop had no significant effect on the total yield of dry herb of pharmacopœial quality obtained for the season.

The alkaloid content was in all cases higher than the minimum required by the U. S. Pharmacopœia, ranging from 0.44 per cent to 0.89 per cent. When the crop was harvested twice, namely, on July 28 and on September 15, there was very little difference in this respect. When it was harvested three times the herb obtained on July 12 and on August 19 differed only moder-

TABLE I.—YIELD AND QUALITY OF HERB OBTAINED FROM BELLADONNA IN THE FIRST SEASON WHEN HARVESTED ONCE, TWICE AND THREE TIMES

| Treatment | Replicated Plot Number | Date of Harvesting | No. of Plants Growing | No. of Plants Cut[a] | Height of Plants, In. Tallest | Height of Plants, In. Shortest | Yield of Herb[b] Green Weight, Oz. | Moisture, % | Dry Weight, Gm. | Total Dry Weight per Plant Cut, Gm. | Total Dry Weight per Plant per Season, Gm. | Calculated Weight of Dry Herb per Acre, Lb.[c] | Percentage of Alkaloids In Herb at Each Harvest | Percentage of Alkaloids Av. for Season[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B*—Harvested once | 1 | Sept. 2 | 73 | 73 | 26 | 14 | 1136 | 80.3 | 5957 | 81.6 | 81.6 | 1273 | 0.66 | 0.66 |
| | 2 | Sept. 2 | 81 | 81 | 25 | 12 | 1024 | 83.0 | 4947 | 61.1 | 61.1 | 1057 | 0.76 | 0.76 |
| | 3 | Sept. 2 | 91 | 91 | 27 | 12 | 976 | 82.9 | 4739 | 52.1 | 52.1 | 1013 | 0.78 | 0.78 |
| | 4 | Sept. 2 | 96 | 96 | 22 | 10 | 816 | 83.7 | 3774 | 39.3 | 39.3 | 806 | 0.70 | 0.70 |
| *C*—Harvested twice | 1 | July 28 | 73 | 73 | 26 | 7 | 680 | 84.1 | 2950 | 40.4 | | Av. 1037 | 0.66 | |
| | | Sept. 15 | 72 | 72 | 28 | 10 | 1038 | 87.6 | 3639 | 50.5 | | | 0.61 | |
| | | | | | | | | | | | 90.9 | 1408 | | 0.63 |
| | 2 | July 28 | 90 | 89 | 24 | 8 | 577 | 82.7 | 2726 | 30.6 | | | 0.66 | |
| | | Sept. 15 | 92 | 90 | 23 | 11 | 688 | 86.9 | 2550 | 28.3 | | | 0.62 | |
| | | | | | | | | | | | 58.9 | 1127 | | 0.64 |
| | 3 | July 28 | 86 | 82 | 25 | 6 | 626 | 82.2 | 2868 | 35.0 | | | 0.66 | |
| | | Sept. 15 | 86 | 86 | 30 | 12 | 1060 | 86.9 | 3935 | 45.8 | | | 0.63 | |
| | | | | | | | | | | | 80.8 | 1454 | | 0.64 |
| | 4 | July 28 | 80 | 79 | 21 | 9 | 443 | 83.1 | 2086 | 26.4 | | | 0.60 | |
| | | Sept. 15 | 80 | 80 | 28 | 8 | 736 | 85.5 | 3022 | 37.8 | | | 0.64 | |
| | | | | | | | | | | | 64.2 | 1091 | | 0.62 |
| | | | | | | | | | | | | Av. 1270 | | |
| *D*—Harvested three times | 1 | July 12 | 81 | 77 | 22 | 6 | 204 | 89.1 | 627 | 8.1 | | | 0.71 | |
| | | Aug. 19 | 81 | 81 | 24 | 11 | 628 | 86.5 | 2398 | 29.6 | | | 0.81 | |
| | | Sept. 28 | 81 | 81 | 27 | 9 | 632 | 84.1 | 2856 | 35.3 | | | 0.50 | |
| | | | | | | | | | | | 73.0 | 1257 | | 0.65 |
| | 2 | July 12 | 71 | 68 | 22 | 4 | 247 | 88.3 | 845 | 12.4 | | | 0.72 | |
| | | Aug. 19 | 71 | 71 | 24 | 10 | 532 | 85.5 | 2178 | 30.7 | | | 0.81 | |
| | | Sept. 28 | 71 | 71 | 26 | 10 | 692 | 86.7 | 2612 | 36.8 | | | 0.44 | |
| | | | | | | | | | | | 79.9 | 1204 | | 0.62 |
| | 3 | July 12 | 84 | 80 | 22 | 4 | 361 | 89.2 | 1114 | 13.9 | | | 0.80 | |
| | | Aug. 19 | 84 | 82 | 18 | 7 | 394 | 85.5 | 1618 | 19.7 | | | 0.78 | |
| | | Sept. 28 | 84 | 84 | 21 | 10 | 612 | 85.7 | 2475 | 29.5 | | | 0.52 | |
| | | | | | | | | | | | 63.1 | 1113 | | 0.66 |
| | 4 | July 12 | 80 | 76 | 18 | 4 | 160 | 87.8 | 551 | 7.3 | | | 0.89 | |
| | | Aug. 19 | 81 | 71 | 15 | 6 | 192 | 82.3 | 805 | 11.3 | | | 0.77 | |
| | | Sept. 28 | 81 | 81 | 18 | 11 | 412 | 86.6 | 1571 | 19.4 | | | 0.61 | |
| | | | | | | | | | | | 38.0 | 625 | | 0.71 |
| | | | | | | | | | | | | Av. 1050 | | |

[a] Some plants were too small to be cut on some of the dates.
[b] Under treatment *B* the dried herb obtained contained some large stems which were removed. The figures given in this column represent the weight of drug of U. S. P. requirements.
[c] These per acre yields are calculated on the basis of the weight of herb obtained from each replicated plot having the number of plants shown and not on the basis of a full stand.
[d] Percentage of alkaloids in all the herb obtained from a replication during the season.

TABLE II.—EFFECT OF MULCHING WITH STRAW AND THE FREQUENCY OF HARVESTING ON THE WINTER SURVIVAL OF BELLADONNA

| Frequency and Time of Harvest in 1943 and Mulching Treatments | Replications I: No. of Live Plants on Nov. 12, 1943 | I: Apr. 26, 1944 | I: Survival, % | II: Nov. 12, 1943 | II: Apr. 26, 1944 | II: Survival, % | III: Nov. 12, 1943 | III: Apr. 26, 1944 | III: Survival, % | IV: Nov. 12, 1943 | IV: Apr. 26, 1944 | IV: Survival, % | Survival in All Replications: Nov. 12, 1943 | Survival in All Replications: Apr. 26, 1944 | Survival in All Replications: Survival, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A*—Not harvested | | | | | | | | | | | | | | | |
| (*a*) Cut Nov. 12[a] | | | | | | | | | | | | | | | |
| (1) Naked | 39 | 9 | 23.1 | 35 | 12 | 34.3 | 46 | 28 | 60.9 | 44 | 12 | 27.3 | 164 | 61 | 37.2 |
| (2) Mulched | 33 | 33 | 100.0 | 37 | 37 | 100.0 | 41 | 41 | 100.0 | 46 | 45 | 97.8 | 157 | 156 | 99.4 |
| (*b*) Uncut | | | | | | | | | | | | | | | |
| (1) Naked | 77 | 51 | 66.2 | 85 | 76 | 89.4 | 81 | 78 | 96.3 | 92 | 51 | 55.4 | 335 | 256 | 76.4 |
| *B*—Harvested once (Sept. 2) | | | | | | | | | | | | | | | |
| (*a*) Cut Nov. 12[a] | | | | | | | | | | | | | | | |
| (1) Naked | 44 | 11 | 25.0 | 44 | 23 | 52.3 | 47 | 8 | 17.0 | 47 | 4 | 8.5 | 182 | 46 | 25.3 |
| (2) Mulched | 36 | 35 | 97.2 | 39 | 36 | 92.3 | 39 | 39 | 100.0 | 43 | 42 | 97.7 | 157 | 152 | 96.7 |
| (*b*) Uncut | | | | | | | | | | | | | | | |
| (1) Naked | 40 | 12 | 30.0 | 40 | 19 | 47.5 | 43 | 8 | 18.6 | 43 | 22 | 51.2 | 166 | 61 | 36.7 |
| (2) Mulched | 43 | 42 | 97.7 | 49 | 48 | 97.9 | 45 | 44 | 97.7 | 50 | 49 | 98.0 | 218 | 214 | 98.2 |
| *C*—Harvested twice (July 28; Sept. 15) | | | | | | | | | | | | | | | |
| (1) Naked | 66 | 25 | 37.9 | 82 | 14 | 17.1 | 81 | 0 | 0.0 | 80 | 6 | 7.5 | 309 | 45 | 14.6 |
| (2) Mulched | 76 | 69 | 90.8 | 88 | 84 | 95.4 | 82 | 67 | 81.7 | 81 | 76 | 93.8 | 327 | 296 | 90.5 |
| *D*—Harvested three times (July 12; Aug. 9; Sept. 28) | | | | | | | | | | | | | | | |
| (1) Naked | 71 | 25 | 35.2 | 79 | 2 | 2.5 | 80 | 8 | 10.0 | 70 | 3 | 4.3 | 300 | 38 | 12.7 |
| (2) Mulched | 89 | 87 | 97.4 | 76 | 70 | 92.1 | 92 | 78 | 94.6 | 90 | 87 | 96.7 | 347 | 322 | 92.8 |

[a] On Nov. 12 the plants that were not harvested at all during the summer were 2 feet tall and bore much fruit. Those that were harvested once (Sept. 2) were from 6 to 12 inches tall. In each case two of the rows in these plots were cut on Nov. 12 after a killing frost and one row left naked and the other mulched. All mulch applied on Nov. 12.

ately in alkaloid content with no consistent trend. However, the herb obtained from the last harvest, September 28, was in all four replications substantially lower in alkaloid content, although well above the minimum required.

The information obtained with respect to the effect of frequency of harvesting and mulching with straw on the winter survival of one-year-old belladonna plants is conclusive. From the data in Table II a number of comparisons are possible between the survival of plants not harvested or harvested has no significant effect on survival when late top growth is not removed and straw mulch is used, the survival in all cases being above 90 per cent.

(*c*) The effect on survival of removing top growth late in fall from plants not harvested during the season and not mulched is highly significant. When the tops were not removed the survival was 76.4 per cent as compared with 37.2 per cent when the tops were removed.

(*d*) Highly significant benefit is derived from mulching plants not harvested during

Fig. 1.—Experimental plots of belladonna photographed Sept. 7, 1943 showing four blocks of four plots each. In block 1 in foreground the four rows in the plot at the right were harvested Aug. 19; (first harvest on July 12); the four rows in the next plot were harvested July 28; the four rows in the next plot had not been harvested and the last four rows had been harvested on Sept. 2 and had not made any new growth.

vested one, two or three times during the growing season, mulched with straw or left unmulched, and having all late top growth left on or removed. When the data are analyzed by the variance method several conclusions are possible:

(*a*) Harvesting of the plants during the growing season results in a significant loss of plants by winter killing as compared with the loss sustained by plants that are not harvested, when no growth is removed late in the season and no straw mulch is used. The survival in the case of the latter was 76.4 per cent whereas when the plants were harvested, respectively, one, two or three times the survival was 36.7, 14.6 and 12.7 per cent. The loss of plants is not significantly affected by the frequency of harvesting. The foregoing figures must be considered in connection with other data in Table II, which show that the loss of plants in the several replications varied greatly.

(*b*) The number of times the plants are the season when the tops are removed from such plants at the time of mulching. Such plants when mulched showed a survival of 99.4 per cent but only 36 per cent survived when no mulch was used.

The effect of harvesting on the vigor of the plants the following spring was clearly indicated. On April 26 when the straw mulch was removed the largest plants in each block were in the rows in which the plants had not been harvested during the growing season but cut 6 inches from the ground the day after the first killing frost and then mulched. These plants were up to 15 inches high. The new growth had obviously emerged sooner and grown faster than in any other case. No mulch was applied to unharvested plants without first removing the tops because of the difficulty of spreading the straw evenly under such conditions, but it may be assumed that removal of the tops serves no purpose other than facilitating the spreading of the mulch.

From the foregoing conclusions certain generalizations may be made with regard to cultural and harvesting practices that will insure the largest survival of one-year-old plants. When grown only for seed or under circumstances where a harvest in the second season is preferred to one in the first season the plants may be cut late in the fall about 6 inches from the ground and covered with 8 to 12 inches of loose straw. The cutting of the plants is necessary only to facilitate placing of the straw. If a maximum harvest in the first year is desired, the crop may be cut one or more times and a similar mulch applied late in the fall. Under such conditions a survival of 90 per cent or more may be expected when from one to three harvests are made. No information was derived from this experiment regarding the relative effect of more frequent cutting.

Plants that are harvested one or more times and are left unprotected generally do not survive in sufficient number to justify retention of the field for a second-year crop. Under conditions greatly different from those under which these tests were made, the results might be somewhat different but on the whole they are in accord with observations made in commercial fields in regions with cold winters in recent years and it is believed that the information is applicable to belladonna culture in any of the eastern states where the crop has been grown successfully.

## SUMMARY

A replicated plot experiment was conducted with belladonna to study the effect of certain harvesting and mulching practices on the yield and the winter survival of the plants. The calculated total per acre yields obtained by harvesting the plants one, two and three times were 1037, 1270 and 1050 pounds, respectively, of dry herb of U. S. Pharmacopœia quality. Analysis of the data by the variance method shows that there were no significant differences between these yields. The hours of labor required to harvest the crop once are probably less than when it is harvested several times but, on the whole, several harvests spread the labor requirements and permit the use of drying facilities of more limited capacity. The herb obtained by cutting the crop several times contains a larger proportion of leaves and less of large stems and green and ripe fruit than that resulting from a single harvest in late summer. The removal of large stems which is usually necessary when the crop is harvested only once is an additional expense which must be considered.

The alkaloid content was in all cases well above the minimum required by the U. S. Pharmacopœia. The herb cut on September 28, after two previous harvests, was lowest in alkaloid content.

The data on winter survival as shown in Table II permits some definite conclusions concerning the effect of certain practices on the loss of plants under the particular conditions under which the plants were grown. Analysis of the data by the variance method indicates which practices are definitely harmful and to what extent harvesting the plants during the growing season and cutting off all top growth late in fall affect the survival and how much the winter loss can be reduced by the use of a straw mulch under various conditions. Following are the several conclusions that are possible: A large loss results if the plants have been harvested one, two or three times during the growing season and no straw mulch has been used as compared with the loss under the same conditions when the plants have not been harvested. If the top growth is not removed late in the fall, and mulch is applied, the survival is high regardless of whether the plants were harvested one, two or three times during the growing season. The removal of all top growth late in the fall from plants not harvested during the growing season resulted in highly significant losses. If plants so treated are mulched the benefit is highly significant. Plants not harvested start to grow earlier the following spring and grow faster than those harvested one or more times.

From these several conclusions it is clear that under the conditions of this experiment the winter loss of belladonna plants after the first season is due in varying degree to (*a*) harvesting of the crop once or oftener, (*b*) removing all top growth late in the fall. These losses can be prevented to a large extent by protecting the plants with a straw mulch.

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